SYMMETRICA is involved in leaf development and stem cell fate

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DECLARATION

I declare that this is my own work and that contributions made by other parties are acknowledged.

CONTENTS

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ABSTRACT

ASYMMETRIC LEAVES1 (*AS1*) encodes a MYB transcription factor that is expressed in lateral organs of *Arabidopsis* where it excludes expression of meristem promoting *knox* genes. This is unlikely to be the only function of *AS1* as reducing *knox* expression does not suppress the *as1* mutant phenotype. To identify additional targets of *AS1*, gain-of-function and loss-of-function mutant screens were used to identify modifiers of the *as1* mutant phenotype.

A gain of function mutation in the *PTL* gene was isolated as a potential suppresser of the *as1* mutant phenotype in petals. Further genetic analysis suggested that *AS1* and *PTL* are unlikely to act in same pathway.

symmetrica (*sym*), a complete suppresser of *as1*, was isolated in loss-of-function screens. Expression analysis of *knox* expression in lateral organs of *sym* mutants suggests that *SYM* is required for *knox* upregulation in *as1* mutant lateral organs. *AS2*, which is thought to act in a similar position in development to *AS1* also appeared to interact with *SYM* as *as2* mutants were also suppressed in its absence. Triple mutant analysis indicated that *SYM* may work in a similar position in development to *SE*, a previously described partial suppresser of *as1*.

In the absence of the *knox* gene *STM*, formation of a functional SAM during embryogenesis is prevented due to ectopic expression of *AS1* and genes that specify lateral organ fate throughout the apex. However, *as1-1*

stm-1 double mutants form a functional SAM as lateral organ fate signals are reduced due to the lack of *AS1*. The SAM of *as1-1 sym stm-1* triple mutants is arrested, indicating a redundant role for *SYM* in meristem function.

sym also modified mutants at the *PNH* locus, suggesting an interaction with the post translational gene silencing machinery.

SYM was mapped to an interval of 120 Mb linked to *AS1* on the long arm of chromosome 2.

1.0 INTRODUCTION

1.1 *Arabidopsis thaliana* is a model system for plant development

Arabidopsis thaliana is a small herbaceous annual member of the Brassicaceae, thought to have its origins in Eurasia. It has been utilised as a model biological system since 1943 when its advantages in classical genetic experiments were described. It is small and has a generation time of about six weeks under optimal growth conditions. It is easily crossed but is naturally self-fertile and tends not to open-pollinate. It has a small genome size (~125 megabases) and a small chromosome number (2n = 10) and produces up to 10,000 seed per plant which are easy to mutagenise by X-irradiation or treatment with mutagens (Bowman, 1993; Howell, 1998a).

Development of the aerial parts of higher plants requires meristems. Situated at the apex of the shoot above the most recently formed leaf, they continuously initiate new organs at their flanks while maintaining a group of self-perpetuating stem cells at their centre. The genetic controls required for this balance between pleuripotency at the centre of meristems, and controlled loss of pleuripotency at meristem flanks is an expanding topic of investigation and a more detailed picture is beginning to emerge. This piece of work describes an investigation into the control of plant organogenesis at the shoot apex using *Arabidopsis* as a model organism, and attempts to add detail to the current model. A general overview of *Arabidopsis* development is followed by a description of genetic mechanisms that control organ initiation, and maintenance of the stem cell population at the shoot apex.

1.2 Embryo and seedling development in *Arabidopsis thaliana*

The life cycle of *Arabidopsis* begins with fertilisation of an egg leading to zygote formation. In angiosperms a double fertilisation event is required in which two sperm cells fuse with nuclei in the embryo sac. One sperm cell fuses with the egg cell to create a diploid embryo. The other fuses with the diploid central cell resulting in a triploid nucleus which becomes the first cell of the endosperm - a tissue that will provide nutritional support for the embryo.

The first step to development of a body plan is the development of polarity which is thought to be influenced by maternal tissue. This begins with elongation of the zygote along the presumptive embryo axis prior to the first cell division. The first two cell divisions are transverse, creating a four cell filament, the smallest cell of which, located at the distal end of the filament, will go on to develop into the proembryo following longitudinal and transverse cell divisions (Fig. 1.1). The remaining three cells develop the suspensor, an organ which anchors the embryo to maternal tissue and is thought to have a role in transferring nutrients from maternal tissue to the embryo. The proembyro develops into the globular embryo after the 16 cell stage, when three fundamental cell layers are distinguishable that give rise to the epidermis, ground meristem, and procambium, respectively.

The globular stage embryo enters heart stage with the formation of cotyledons and the axis system. The root apical meristem, responsible for all below ground postembryonic development, and constructed from cells of the

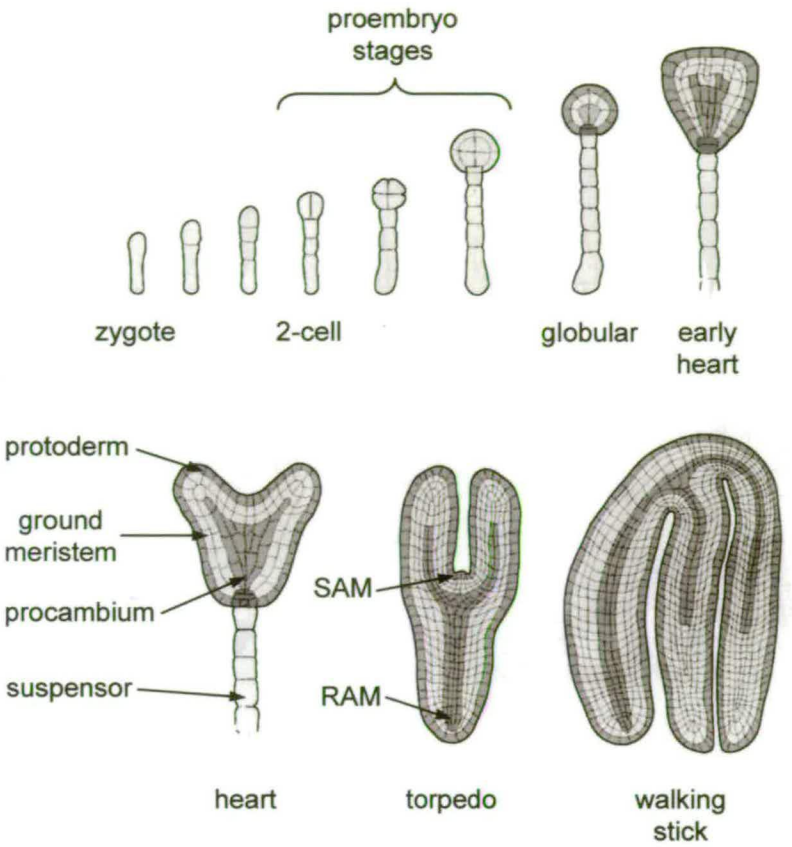


Figure 1.1 Diagrammatic representation of embryo development in *Arabidopsis*.
The zygote undergoes transverse cell divisions to develop a proembryo and a suspensor. Longitudinal and transverse cell divisions of the proembryo result in a globular embryo. Cotyledons begin to emerge at heart stage. Both meristems are present by torpedo stage. Cotyledons are bent over by walking stick stage due to limited space in the developing ovule. (From Howell, 1998)

proembryo and the upper cell of the suspensor, is distinguishable at this stage. Subsequent stages in embryo development reflect shape changes mainly associated with cotyledon size. Torpedo stage follows heart stage, and at the transition between the two, the shoot apical meristem (SAM), responsible for all above ground post embryonic development, becomes histologically distinguishable between the cotyledons. Cells from the three fundamental cell layers are recruited to the SAM and these cell lineages are maintained in the postembryonic shoot. The final stage of embryo development, when the embryo has reached maturity is the walking stick stage where the cotyledons are bent over due to limiting space in the developing ovule.

Development of the seedling occurs at the transition between embryonic and post embryonic development. Seedling development begins below the soil surface. The hypocotyl elongates and pushes the folded cotyledons upward through the soil. Once exposed to light, elongation of the hypocotyl stops and cotyledons green and enlarge. The SAM is activated and the first true leaves of vegetative development are initiated.

1.3 Shoot apical meristem organisation

Meristems are self-renewing structures. They contain small groups of pluripotent stem cells, that divide and give rise to the primary shoot, lateral organs and all postembryonic structures in plants. The constant differentiation of cells in organ formation is balanced by cell divisions and therefore

continuous replenishment of the pluripotent stem cell population. *Arabidopsis*, and all angiosperm shoot apical meristems, are composed of three fundamental cell layers, L1, L2, and L3 that can be traced back to the embryo. The L1 layer gives rise to the epidermis and comprises a sheet of cells in which all divisions are anticlinal and therefore daughter cells remain within the layer (Fig. 1.2). In the middle layer of cells, anticlinal cell divisions predominate, but periclinal cell divisions do occur. The L2 gives rise to sub epidermal tissue such as the cortex, and outer layers of palisade and spongy mesophyll cells in the leaf. The L1 and L2 are sometimes referred to as the tunica layer, while the L3 is referred to as the corpus. In the L3, periclinal cell divisions predominate giving rise to pith in the stem, and inner layers of spongy mesophyll cells and vasculature in leaves (Howell, 1998b; Steeves and Sussex, 1989).

Meristems can also be divided into different zones defined by rates of cell division and differentiation. The central zone (CZ) contains large vacuolated stem cells that divide infrequently. The CZ cells are thought to be progenitors of smaller undifferentiated cells in the surrounding peripheral zone (PZ) which have less vacuole and more cytoplasm than CZ cells. PZ cells divide more rapidly, generating cells used in lateral organ formation. There is continual displacement of cells from the peripheral zone to the flanks of the apex where organs are formed, an area sometimes referred to as the morphogenic zone. The rib zone (RZ) contains similar cells to PZ cells.

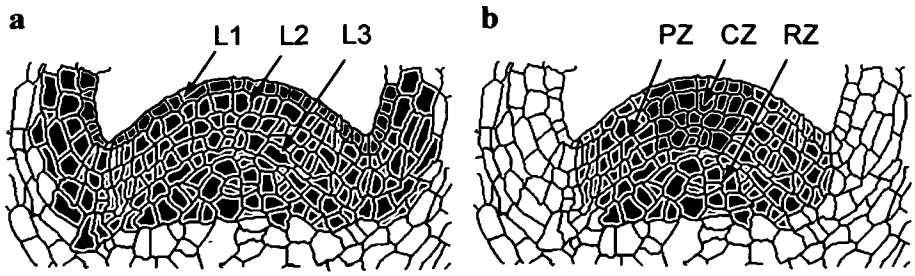


Figure 1.2 layers and zones within the shoot apical meristem.

a The L1 layer (dark blue) gives rise to epidermal tissue. The L2 layer (green) gives rise to sub epidermal tissue. The L3 layer (light blue) gives rise to inner cell layers. **b** The CZ contains large, vacuolated cells that divide slowly. Displacement of older CZ cells into the PZ or RZ is accompanied with physiological changes as cells in the PZ and RZ are cytoplasm rich and divide more rapidly. The PZ gives rise to lateral organs. The RZ gives rise to stem. (Figures adapted from Haecker and Laux, 2001.)

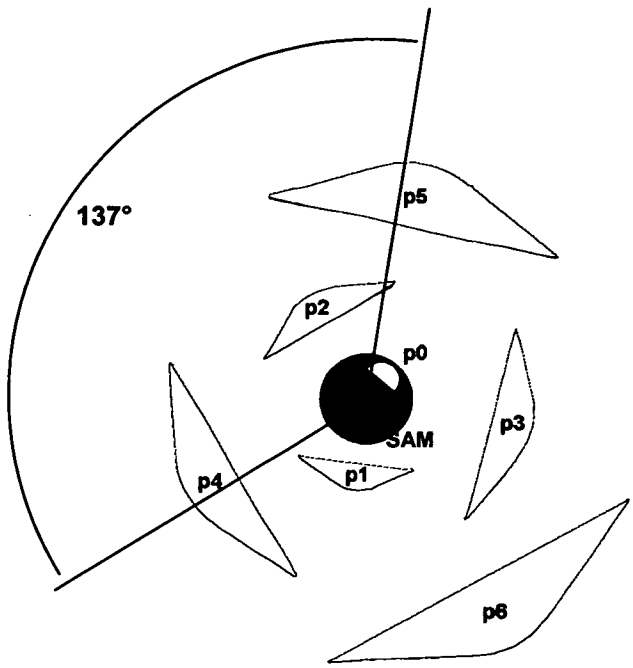


Figure 1.3 Phylotaxy of lateral organs. From the third leaf onward, initiation of *Arabidopsis* lateral organs follows spiral phylotaxy. Successive primordia are initiated at an angle of about 137 degrees in a sequential manner. Younger lateral organs are higher up the shoot. The emerging lateral organ primordium is p0. p1 is younger than p2, p2 is younger than p3, and so on.

Division and elongation of RZ cells is involved in growth of the stem (Howell, 1998a; Steeves and Sussex, 1989)

1.4 Vegetative development

The arrangement of leaves and other lateral organs around the shoot is established in the SAM. The two cotyledons develop opposite each other and the first two rosette leaves form in positions between the cotyledons, opposite each other. From the third leaf onward, initiation of *Arabidopsis* lateral organs follows spiral phylotaxy with successive primordia initiated at an angle of about 137° in a sequential manner (Fig. 1.3). Younger lateral organs are therefore found higher up the shoot. Leaf primordia first emerge as radially symmetric bumps on the side of the SAM. Primordia enlarge and begin to adopt a dorsoventral asymmetry. Trichomes - unicellular hairs of unknown function - become apparent on the distal adaxial leaf surface. Paired leafy appendages, the stipules, enlarge at the base of the primordia. Primordia elongate along their proximodistal axis by cell division. As the lamina extends from the early midrib, development of the leaf itself is marked by cell elongation in addition to cell division. Later, differentiation of mesophyll tissue into palisade and spongy mesophyll is evident as dorsoventral differences continue to emerge. A developmental gradient also exists across the proximodistal axis of the emerging leaf as the proximal part of the leaf contains small, undifferentiated, rapidly dividing cells while more distally cells undertake differentiation and expansion marked by the appearance of

vacuoles. Late in leaf development, cell expansion is responsible for increasing leaf size as first proximodistal leaf expansion, lateral expansion and finally thickening of the lamina cease.

Growth conditions and ecotype affect the number of rosette leaves formed by *Arabidopsis* before entering the reproductive phase. As many as 30, or as few as five leaves may be generated prior to formation of the inflorescence (Bowman, 1993).

1.5 Reproductive development

At the transition to reproductive growth, the vegetative SAM becomes converted directly into an inflorescence meristem and anatomical changes are associated with the conversion. The inflorescence meristem is more convex than the vegetative SAM, due to an increase in RZ size. The RZ becomes more active and forms the longer internodes of the inflorescence stem. The identity of the lateral organs formed on the flanks of the meristem changes as cauline leaves and flowers replace rosette leaves and axillary SAMs, although their positions follow the same spiral phylotaxy. The primary inflorescence develops cauline leaves at basal positions and a potentially indeterminate number of flowers at apical positions. Secondary inflorescence meristems that develop in the axil of each cauline leaf of the primary inflorescence which repeat the pattern of lateral organ development outlined for the primary inflorescence. Tertiary inflorescences arise in the axils of the cauline leaves

on secondary inflorescences. Further inflorescence meristems develop in the axils of the more apical rosette leaves (Bowman, 1993; Howell, 1998a).

At the transition from inflorescence meristem to flower, a transition from indeterminate to determinate growth occurs as floral meristems generate a specific number of organs. The flower usually has, from the outside to its center, four sepals, four petals, six stamens and a gynoecium made up of two carpels. Flower meristems arise on the flanks of the inflorescence meristem and are soon demarcated from the inflorescence meristem. The four sepal primordia are first to appear followed by the petal and stamen primordia together. The floral meristem itself then becomes the gynoecium primordia. Differentiation of sepal primordia occurs once all fifteen primordial that will go on to develop into floral organs have emerged. Differentiation of stamens and carpels follows, and finally petal differentiation occurs (Bowman, 1993; Howell, 1998a).

1.6 Genes that regulate the shoot apical meristem

Maintenance of the stem cell populations in the various zones of the SAM is essential for normal plant development. The *WUSCHEL* (*WUS*) gene of *Arabidopsis*, which encodes a homeodomain protein expressed below the stem cell population at the shoot apex (Mayer *et al.*, 1998), has an important role in regulation of the stem cell population. In *wus* mutants, stem cells differentiate prematurely leading to no recognisable meristem between leaf primordia, and no histological differences between peripheral and central regions within the

apex suggesting a role for *WUS* in maintenance of the stem cell population (Laux *et al.*, 1996). *WUS* is assisted in its role in meristem maintenance by 3 *CLAVATA* (*CLV*) genes, *CLV1*, *2* and *3*, which act together to control the size of the pluripotent stem cell population. In *clv1* mutants, plants produce a much larger SAM in both vegetative and reproductive growth thought to be due to an increase in CZ size resulting from a delay in CZ to PZ transition (Schoof, *et al.*, 2000). In weak mutants, this manifests as increased organ number in the flower. Intermediate and strong alleles cause fasciation and massive over proliferation of the meristem, respectively (Clark *et al.*, 1993). Similar meristem defects occur in *clv2* and *clv3* mutants (Clark *et al.*, 1995; Kayes and Clark, 1998). The *CLV3* gene encodes a secreted signalling molecule expressed in the CZ cells at the shoot apex (Fletcher *et al.*, 1999). Once secreted, the CLV3 protein is thought to bind to and activate a heterodimeric receptor kinase complex made up of the CLV1 and CLV2 proteins (Trotochaud *et al.*, 2000). One of the results of activation of this complex is down regulation of *WUS* expression which presumably leads to reduction in the size of the stem cell population (Brand *et al.*, 2000). Interestingly, *WUS* is likely to act as an activator of *CLV3* suggesting that in a situation where the area of *WUS* expression becomes too large, the *CLAVATA* pathway is upregulated, resulting in *WUS* down regulation. In this way, in *clv* mutants, the SAM becomes enlarged due to inability of mutants to down regulate *WUS* (Brand *et al.*, 2002; Schoof *et al.*, 2000). There are however other factors likely to be involved in *CLV3* upregulation as transgenic plants

that ectopically express *WUS* in leaves do not mis express *CLV3* in leaves (Brand *et al.*, 2002). A likely candidate for *CLAVATA* pathway activation is the *SHOOT MERISTEMLESS* gene, which is required for meristem over proliferation in *clv* mutants (Clark *et al.*, 1996). Transgenic plants that misexpress *STM* in leaves do not ectopically express *CLV* genes, as is the case with ectopic *WUS* expression. Transgenic plants that misexpress both *WUS* and *STM* however, do misexpress *CLV* and develop ectopic meristems (Brand *et al.*, 2002; Gallois *et al.*, 2002; Lenhard *et al.*, 2002).

1.7 *SHOOT MERSTEMLESS* is required for a functional shoot apical meristem

The *SHOOT MERISTEMLESS* (*STM*) gene is required for SAM formation in the embryo and required throughout development for normal SAM function (Endrizzi *et al.*, 1996). In wild-type walking-stick stage embryos, the SAM has already formed, but in plants carrying the strong *stm-1* mutation cells at the apex have cells characteristics of the torpedo stage apex where no SAM is histologically distinguishable (Barton and Poethig, 1993). *STM* expression first appears in one or two cells at the early to mid-globular stage of embryo development. By late globular stage *STM* mRNA appears in a strip across the top half of the embryo, but by heart stage expression is restricted to the notch between the cotyledons where the shoot apical meristem will appear later in development. Expression persists thereafter in the vegetative, auxiliary, inflorescence and floral meristems with the interesting exception of its

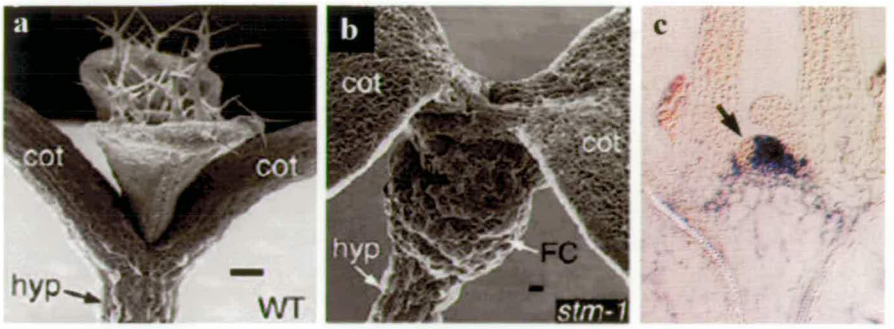


Figure 1.4 *STM* expression and mutant phenotype.
a Wild type plant with two leaves developed from where the cotyledons (cot) meet the hypocotyl (hyp). **b** In *stm-1* mutants, cotyledons fuse (FC), where the SAM normally forms. **c** In vegetative tissues, *STM* RNA is detected in the SAM, except at positions of developing lateral organs (arrow). (**a** and **b** from Barton and Poethig, 1993. **c.** from Endrizzi, *et al.*, 1996.)

absence at positions of developing leaf primordia, floral bud initiation and floral organ initiation (Fig. 1.4). This has suggested that down regulation of *STM* is required for leaf development and lateral organ development in general, indeed this can be considered the earliest known marker for lateral organ initials (Long *et al.*, 1996).

STM is member of a small sub-family (Class I) of ***knotted-1*** like homeobox genes in *Arabidopsis*. Sequence analysis of the *Arabidopsis* genome suggests that only four members exist: *STM*, *BREVIPEDICELLUS* (*BP*; also known as *KNAT1*), *KNAT2* and *KNAT6* (Lincoln *et al.*, 1994b; Long *et al.*, 1996; Semiarti *et al.*, 2001). The founding member of the knox family is a *Zea mays* gene, *KNOTTED-1* (*KN1*), the first homeobox gene identified in the plant kingdom. Like many animal homeobox genes, *KN1* regulates processes that determine cell fate, despite the common ancestor of the two kingdoms being unicellular (Vollbrecht *et al.*, 1991).

1.8 Maize *Knotted-1* mutants have knots associated with lateral veins on leaves

Maize plants carrying dominant *Kn1* mutations develop leaves that show a decrease in overall area, but an increase in blade width. Leaves also develop numerous protuberances termed knots that extend from the abaxial surface of the lamina (Bryan and Sass, 1941). Knots are the result of centres of intercalary meristematic activity that occur along the leaf veins and remain

active throughout the growth of the blade. The continued meristematic activity and the physical restraint of the surrounding non-meristematic tissue gives rise to the protuberances. Knot formation originates in one or several cells overlying a vein. Isolated patches of epidermal cells fail to respond to factors inducing cells to stop dividing. Up to 10 knots per vein are developed, with fewer knots at the leaf margins and near the midvein. The *Kn* blade is further modified by displacement of the ligule from its normal position at the juncture of blade and sheath to a more distal position. The amount of knotting is generally proportional to the amount of ligule displacement (Gelinas *et al.*, 1969). The dominant *Kn1* alleles are gain-of-function mutations which leads to ectopic expression of the gene in leaves and other lateral organs (Sinha *et al.*, 1993).

Loss-of-function *kn1* mutants also have defects that vary with genetic background, but which suggest compromised meristem activity (Kerstetter *et al.*, 1997; Vollbrecht *et al.*, 2000). Weaker alleles cause a reduction in branching from the inflorescence meristem and a number of plants develop two leaves at one node. The extra leaf is narrower than the normal leaf and may be significantly shorter. The production of extra organs as an outcome of the loss of meristem-specific *KN1* expression has been interpreted as resembling the normal events of lateral organ development. Down regulation of *KN1* expression at the SAM boundary may represent a key signal for cells to respond to factors that promote cellular differentiation. In the loss-of-function mutants this boundary may no longer be strictly maintained. Cells in

the SAM that would, in the presence of KN1, resist signals to differentiate and remain meristematic be induced to form organ primordia (Kerstetter *et al.*, 1997). Vollbrecht *et al.* (2000) described *kn1* loss-of-function mutants in an inbred genetic background which had a naturally small meristem size. These plants fail to maintain the meristem such that three leaves at most are developed. The authors interpret this as evidence of a peripheral zone function for *KN1* and suggest that this function is to specify the PZ boundary, co-ordinate the contribution of CZ cells to the PZ, or specify an indeterminate fate to PZ cells. The expression pattern of *KN1* and other *knox* family members in maize, support this PZ function. In general the genes are strongly expressed around the SAM, moderately or weakly expressed in the embryo and other restricted tissue and absent or expressed at low levels in differentiated tissue (Jackson *et al.*, 1994; Kerstetter *et al.*, 1994).

1.9 *brevipedicellus* mutants form a functional SAM but have altered inflorescence architecture

BREVIPEDICELLUS (*BP*) is probably the most similar of the *Arabidopsis* knox genes to *KNOTTED1* in sequence. In its absence, leaves are initiated at a slightly faster rate than the Lansberg *erecta* (L*er*) wild-type, and the floral meristem is initiated slightly earlier. This suggests a role for *BP* in regulating rates of leaf initiation and floral transition, but if it is involved in meristem activity *BP* must, unlike *STM*, act redundantly (Douglas *et al.*, 2002). Greater differences in *bp* mutants become evident at the time of bolting. Bending of

the stem takes place at nodes. Stripes made up of cell files with defects in epidermal cell differentiation extend down the stem positioned over vascular bundles. Floral buds are produced at the apex in a more compact arrangement and begin pointing down early in their development leading to downward-pointing siliques. At all floral nodes, pedicels demonstrate a large reduction in length and development of these organs is much slower. Epidermal cell differentiation never reaches completion on the abaxial side and both epidermal and cortical abaxial cells do not elongate as wild-type. Organs derived from the floral meristem are normal with the exception of the style which loses radial symmetry. Arrangement of sigmatic papillae is altered as a result (Douglas *et al.*, 2002; Venglat *et al.*, 2002).

BP is expressed in the SAM, localised to the PZ and RZ, and absent from the CZ, mirroring maize *KN1* expression. *BP* transcripts are also observed immediately below the SAM, and below developing leaf primordia. Developing primordia themselves are devoid of *BP* mRNA. In the inflorescence, expression is also detectable in the cortex of the pedicel (Lincoln *et al.*, 1994b). Ectopic *BP* expression results in altered leaf morphology. When *BP* is driven from the *35S* promoter, profound asymmetrical lobeing occurs in both rosette and cauline leaves. Early stages of leaf development are identical to wild-type until initiation of serrations. In plants misexpressing *BP*, serrations develop into prominent primordia that go on to from the characteristic lobes. Often second order lobes appear on lobes initiated after the first pair and stipules develop at the leaf margin. Laminae

also appear curled and wrinkled and, in extreme cases, the petiole is difficult to distinguish from the upper surface of the leaf blade (Chuck *et al.*, 1996; Lincoln *et al.*, 1994b). Palisade cells are not developed but an extra layer of small tightly packed mesophyll cells with reduced air spaces are present. Alteration of venation pattern occurs, as the vascular system of *35S::BP* mutant leaves is no longer closed and continuous and the midvein has approximately double the number of vascular elements. Ectopic meristems form in rows at the sinuses of rosette and cauline leaves on the adaxial surface directly over a vein, but are mostly not maintained. *STM* expression is detected in ectopic meristems following establishment of tunica-corpus organisation (Chuck *et al.*, 1996).

1.10 *bp* mutant phenotypes are enhanced by *erecta*.

The *erecta* mutation causes increased stem thickness and reduced elongation of internodes and pedicels, resulting in a more erect appearance, and silique morphology is also altered (Torii *et al.*, 1996). *ERECTA* encodes a putative receptor that is expressed at low levels in internodes and pedicels where most morphological differences are observed in its absence. Expression is also low in leaves but high expression occurs in peripheral and central zones of the SAM, inflorescence and floral meristems (Yokoyama *et al.*, 1998).

In *bp er* double mutants all aspects of the *bp* phenotype are enhanced. Internode elongation is significantly reduced. Stripes of cell files with epidermal defects extend further down the stem. Bends at the nodes are more

extreme and silques point down at a more severe angle suggesting that *BP* and *ER* have overlapping functions in regulation of plant architecture (Douglas *et al.*, 2002).

1.11 Plants over expressing *KNAT2* reveal roles for *knox* genes in carpel development

KNAT2 is expressed in vegetative tissue in a similar manner to *BP*. It is expressed in the SAM and downregulated in primordia of lateral organs. Following transition to reproductive development expression is reactivated in a manner consistent with a role in carpel development, specifically, expression extends to the recepticle and the developing pistil (Dockx *et al.*, 1995). Ectopic *KNAT2* expression in flowers in a L*er* genetic background causes inhibition of the elongation of floral organs, with the exception of the carpel. Moreover, ectopic carpel-like structures complete with stigmatic papillae develop from placental tissue in place of ovules following embryo sac degeneration. Inside these ectopic carpels, secondary carpel-like structures develop. This homeotic conversion of ovules to carpels is further supported by altered expression of *AGAMOUS* (*AG*), a C function gene. *AG* is normally expressed in the third and fourth whorls of young flowers but strong expression becomes restricted to the stamens, stigma and nectaries of the carpels later in development with weak expression maintained in the carpel walls. In plants where *KNAT2* is overexpressed, strong *AG* expression is maintained over a longer period in the pistil and in the ovules. The

conversion of ovules to carpels is not, however, dependent on the presence of *AG* (Pautot *et al.*, 2001). *KNAT2* is unlikely to be the only *knox* gene involved in carpel development as the carpel constitutes a specialised meristematic tissue that produces ovules (Sessions and Zambryski, 1995) and *STM* is expressed strongly in the floral meristem where *AG* is active (Long *et al.*, 1996).

1.12 The *cup-shaped cotyledon* mutant is phenotypically similar to *stm*

In the absence of *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUP-SHAPED COTYLEDON2* (*CUC2*) gene activities, the *Arabidopsis* embryo fails to develop a shoot apical meristem. Large, highly vacuolated cells are present in the position where the characteristic small cells of the SAM normally form, suggesting that stem cells have been replaced with cells showing organ fate. *cuc1 cuc2* double mutants also fail to separate cotyledons at the margins resulting in a cup-like structure and *CUC* genes are also required for normal separation of some floral organs (Aida *et al.*, 1997) suggesting a wider role in setting boundaries. Interestingly, the *cuc1 cuc2* double mutant phenotype has striking similarities to strong *stm* mutants. Inability to form a functional meristem and fusion of the cotyledons (Barton and Poethig, 1993; Long *et al.*, 1996) are common features. In *stm* mutants cotyledon fusion is less severe and is restricted to regions close to the hypocotyl and does not expand to the lamina. The failure to properly separate cotyledons in *stm* mutants is similar

to cotyledon fusion observed in a small percentage of single *cuc1* or *cuc2* mutants which occurs down one side of the cotyledon, in comparison to fusion of both sides in double mutant cotyledons (Aida *et al.*, 1997).

Both *CUC* genes encode members of a protein family that appears only in plants. All have a number of serine rich regions, and a highly conserved C-terminal. *CUC1* and *CUC2* bear significant similarity to the Petunia *NO APICAL MERISTEM* gene (Aida *et al.*, 1997; Takada *et al.*, 2001) which is required for normal separation of cotyledons and development of a functional SAM (Souer *et al.*, 1996). *CUC1* and *CUC2* are both expressed prior to *STM* expression in globular stage embryos. *CUC1* mRNA is detected in two areas of the upper half of the embryo adjacent to the presumptive site of SAM initiation (Takada *et al.*, 2001), whereas *CUC2* is expressed in the site of presumptive meristem initiation itself (Aida *et al.*, 1999). By late early heart stage, both *CUC* genes are expressed in a stripe between the cotyledons in a similar fashion to *STM* (which appears at late globular stage) a pattern that is maintained until the SAM becomes clonally distinct at late torpedo stage. Expression of the *CUC* genes then begin to differ from that of *STM* (which persists only in the SAM) becoming restricted to the boundary between the cotyledon primordia and the SAM (Aida *et al.*, 1999; Takada *et al.*, 2001).

1.13 *CUC1* and *CUC2* are required for normal accumulation of *STM* in developing embryos

Plants homozygous for *stm*, *cuc1*, and *cuc2* mutations appear as *cuc1 cuc2* double mutants, suggesting that the CUC activity might be required for *STM* action. Furthermore, *STM* mRNA is not detected in *cuc* double mutant embryos suggesting that the *CUC* genes activate *STM* either directly or indirectly. The *CUC* genes clearly act redundantly on STM, as *STM* transcripts accumulate normally in *cuc1* or *cuc2* single mutants, although both *stm-1 cuc1* and *stm-1 cuc2* double mutants show greater fusion of cotyledons that the *stm-1* single mutants (Aida *et al.*, 1999). In *stm* mutants, the spatial expression patterns of the *CUC* genes are also altered. *CUC1* expression is present throughout the boundary region of the cotyledons, as would be expected in the absence of an SAM (Takada *et al.*, 2001). *CUC2* mRNA localisation, however, is slightly different in that expression is restricted to a spot in either the centre of the embryo between the cotyledons, or at a lateral position, or both lateral positions (where the first true leaves would normally form) suggesting a role in setting boundaries required for cotyledon separation and SAM initiation (Aida *et al.*, 1999).

Perhaps the strongest evidence for *CUC* being required for *STM* expression comes from plants showing ectopic CUC activity. These plants, which express the *CUC1* from the *35S* promoter, have lobed cotyledons and the characteristic lobed leaves of *knox* mis-expression. Both *STM* (Takada *et al.*, 2001) and *KNAT1* (Hibara *et al.*, 2002) are mis-expressed in the cotyledons of

plants carrying a transgene and adventitious shoots, and in some cases leaves form on the adaxial surface of the cotyledons near vascular bundles. In some cases ectopic meristems go on to develop rosette leaves (Takada *et al.*, 2001).

1.14 *SHOOT MERISTEMLESS* is required for exclusion of *ASYMMETRIC LEAVES1* from the shoot apical meristem

Aside from its interactions with other genes required for normal meristem function, *STM* maintains the meristem by excluding the expression of *ASYMMETRIC LEAVES1* (*AS1*), a gene involved in leaf development from the SAM. *AS1* is first expressed during embryo development in two sub epidermal layers that correspond to cotyledon initials and expression is maintained in cotyledons throughout development. In *stm* mutants however, the region of *AS1* expression is expanded to include the developing SAM (Fig. 1.5). The suggestion that *STM* is required to negatively regulate *AS1* in the apex is supported by *as1-1 stm-1* double mutants which produce a functional meristem. In *as1* mutants, *STM* is no longer required to exclude AS1 activity, and therefore lateral organ fate, from the SAM, and the pluripotent stem cell population is maintained (Byrne *et al.*, 2000). It has also been suggested that *STM* plays a part in negative regulation of other genes that specify lateral organ fate from the SAM, including *YABBY3* and *FILAMENTOUS FLOWER* genes and is therefore likely to represent a general mechanism for lateral organ suppression (Kumaran *et al.*, 2002).

1.15 *AS1* is required for normal leaf development.

asymmetric leaves1 is a classical mutation in *Arabidopsis* required for specification of leaves and structures homologous to leaves. In its absence development of cotyledons, leaves and floral organs are disrupted. Leaves, both rosette and cauline demonstrate clearly altered morphology (Tsukaya and Uchimiya, 1997). Laminae become subdivided into prominent outgrowths or lobes which form in a bilaterally asymmetric manner. Early rosette leaves have few lobes located close to the stem and in some plants, the first set of leaves are joined to the petiole below the lamina rather than at its end (Sun *et al.*, 2002). Leaf margins have more cell files than wild-type and that are discontinuous in sinuses due to formation of a distinctive notch (Ori *et al.*, 2000). The strongest allele, *as1-magnifica*, also develops ectopic meristems at the sinuses (Byrne *et al.*, 2000). Later leaves have more lobes that extend to more distal positions on the leaf. Cauline leaves are absent at the site of some lateral inflorescences, but are lobed where present. *as1* mutants have early opening flowers which lack correct elongation of sepals, petals and stamens. In some flowers gynoecia consist of more than two carpels. All lateral organs lack vasculature with correct levels of organisation (Sun *et al.*, 2002).

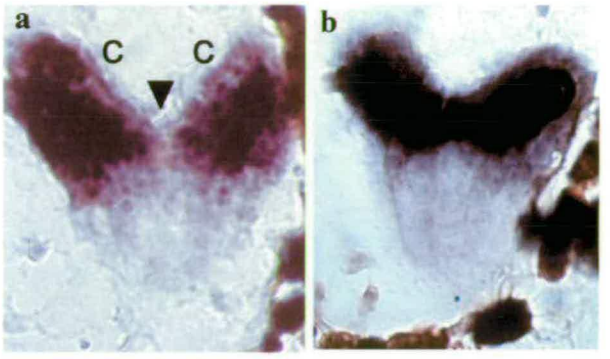


Figure 1.5 Expression of *AS1* in wild type and *stm-1* mutant embryos.
a In wild type, *AS1* RNA is present in the cotyledons (c) and absent from the presumptive SAM (arrow). **b** In *stm-1* mutants *AS1* is expressed throughout the apex including the SAM. (**a** and **b** from Barley, 2001)

1.16 Homologues of *AS1* in *Antirrhinum* and maize.

The *PHANTASTICA* (*PHAN*) gene from *Antirrhinum majus*, and *ROUGH SHEATH2* (RS2) from maize are homologous to *AS1* (Byrne *et al.*, 2000; Timmermans *et al.*, 1999). *phantastica* (*phan*) mutant leaves are defective in several aspects of leaf development such as lateral expansion of the lamina, dorsoventrality and proximal-distal patterning. The *phantastica* mutant phenotype includes formation of heart-shaped leaves below the fifth node similar to *as1* mutant rosette leaves from *Arabidopsis*. *PHAN* is also needed for maintenance of SAM stem cell activity, which would seem to indicate that lateral organ identity is a requirement for apical meristem activity in *Antirrhinum* development (Waites and Hudson, 1995).

rs2 mutants have a variety of phenotypes which are background dependent. The most striking phenotype is formation of bladeless leaves. Other leaf phenotypes include development of leaves with multiple midribs, narrow leaves, leaves with multiple or excessively large midribs or wider leaves with an increased number of veins. Disruption of the blade-sheath boundary occurs in all mutants and is background independent. Dwarf phenotypes and fused nodes occur in some genetic backgrounds (Schneeberger *et al.*, 1998).

PHAN works as a dorsalising factor. In mutant plants, tissues associated with the dorsal part of the wild-type leaf can be replaced by tissues with ventral characteristics. As a result, leaves above the fifth node are typically needle-like, lacking lamina and all cell types associated with the dorsal region of the wild-type leaf. Other mutant leaves where lamina are present

characteristically contain patches of ventral tissue, including stomata on their dorsal surface (Waites and Hudson, 1995). *rs2* mutants on the other hand are considered to have features consistent with a proximalisation of the leaf. Cells in the distal parts of leaves take on characteristics normally only present at positions close to the stem. The function of *RS2* has therefore been interpreted as providing or responding to proximal information (Schneeberger *et al.*, 1998; Tsiantis *et al.*, 1999).

1.17 *PHAN* and *RS2* are expressed in similar domains.

PHAN expression is specific to initials and early primordia of all lateral organs and is expressed in a non-spatially restricted manner in terms of dorsoventral patterning. It therefore must interact with unidentified genes that have spatially restricted activities, in order to exert its role in establishment of dorsoventral axes (Waites *et al.*, 1998). *RS2* is expressed throughout lateral organ initials, but becomes restricted to the vasculature and leaf margins later in development and is restricted to the vasculature alone in mature leaves. Transcript is absent from the meristem (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999).

1.18 *PHAN, RS2* and *AS1* encode Myb proteins.

The N-terminal domain of PHAN is homologous to members of the R2R3-MYB family, common in higher plants, although subtle differences include

two or three additional amino acid residues in the first (R2) MYB repeat of the translated protein, and little conservation in the last third of the second (R3) repeat. The C-terminal domain of the protein shows little similarity to other known proteins with the exception of its likely orthologues (Waites *et al.*, 1998). *RS2* shares 62.9 % identity with *PHAN*, but this is less than the identity shared by *AS1* and *PHAN* (68.8 %; (Byrne *et al.*, 2000; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999).

1.19 AS1 forms a protein complex with ASYMMETRIC LEAVES2 *in vitro*

The *ASYMMETRIC LEAVES2* gene of *Arabidopsis* encodes a member of a protein family, with >30 members in Arabidopsis, characterised by a leucine zipper-like and a $CX_2CX_6CX_3C$ sequence termed the C-motif (Iwakawa *et al.*, 2002b). Formation of leaves with normal bilateral symmetry is prevented in the absence of *AS2*, a common feature with *as1*. Similarities extend to asymmetric lobeing, downward curling, failure to develop a normal midvein, and asymmetric secondary vein formation (Semiarti *et al.*, 2001). *as2* mutants, like *as1* mutants, are suppressers of the *stm* mutant phenotype (Byrne *et al.*, 2002), and although the expression domains of *AS1* and *AS2* are not the same, they overlap. *AS2* is confined to lateral organs, but shows only dorsal expression in developing embryos (Iwakawa *et al.*, 2002b). Studies *in vitro* have shown that the two proteins interact physically (they co-precipitate) suggesting that they work together on downstream targets (Iwakawa *et al.*,

2002a). *AS2* has however been placed upstream of *AS1* in the genetic pathway as *as1 as2* double mutant phenotypes have been described as resembling *as2* mutant characteristics (Ori *et al.*, 2000; Serrano-Cartagena *et al.*, 1999). However, differences in the *as1* and *as2* mutant phenotypes may reflect different genetic backgrounds or severity of mutation.

1.20 *ASYMMETRIC LEAVES1* and *ASYMMETRIC LEAVES2* are required for exclusion of *knox* expression from lateral organs.

AS1, and its functional orthologues, *PHAN* and *RS2*, negatively regulate class 1 *knox* genes in lateral organs (Byrne *et al.*, 2000; Griffith *et al.*, 1999; Ori *et al.*, 2000; Schneeberger *et al.*, 1998; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999; Waites *et al.*, 1998). In *as1* mutants *BP*, *KNAT2* and *KNAT6* are strongly mis-expressed in all developing leaves (Fig. 1.6; Byrne *et al.*, 2000; Ori *et al.*, 2000), and *STM* transcript has been reported to accumulate at low levels in first and second leaves (Semiarti *et al.*, 2001). *as2* mutants are similar in terms of *knox* mis-expression. *KNAT1*, *KNAT2* and *KNAT6* transcripts can be found in *as2* lateral organs, but unlike in *as1*, ectopic *STM* expression has not been reported (Ori *et al.*, 2000; Semiarti *et al.*, 2001). A β-glucuronidase (GUS) reporter under control of the *BP* promoter was not expressed in $AS1^{+}$ wild-type leaves but was expressed in petioles, sinuses and the midvein of both *as1* and *as2* mutants (Ori *et al.*, 2000). This *knox* mis-expression may reflect similarities in phenotype with *35S::BP* mutants. *as1*, *as2* and *35S::BP* mutants all develop asymmetric lobes, defects in vascular

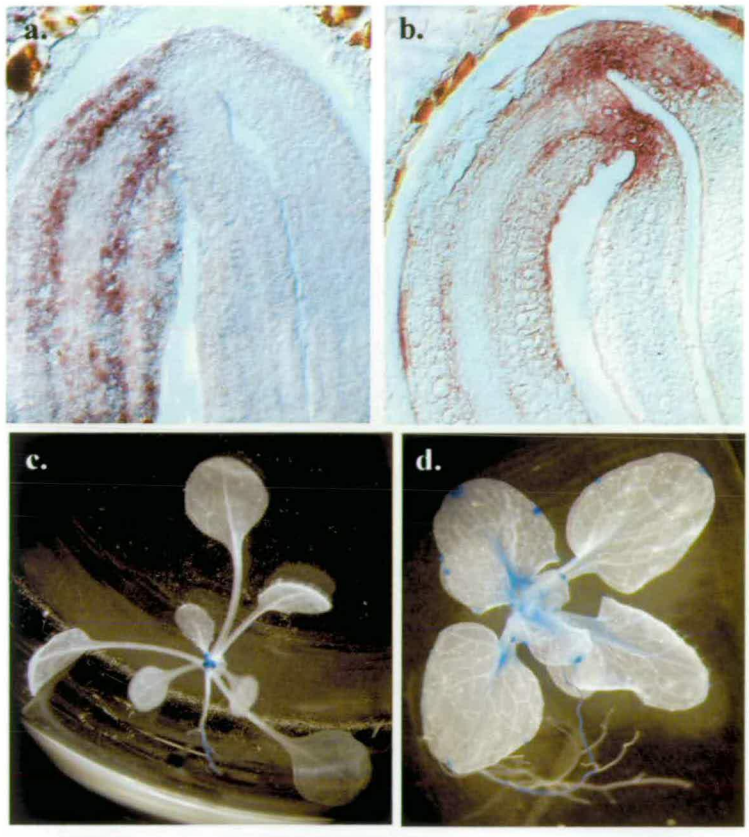


Figure 1.6 *BP* expression in *as1* and wild type embryo and vegetative tissue.

a and **b** *In situ* hybridisation showing *BP* mRNA localisation. **a** *BP* expression in the wild type embryo is present in the morphogenic zone. **b** In *as1* mutant embryos, expression extends to lateral organs. **c** and **d** Plants with *UidA* (GUS) gene driven from the *BP* promoter. **c** In wild type plants, expression is restricted to the shoot apex. **d** In *as1-1* mutants staining is observed in vasculature and sinuses of leaves. (**a** and **b** from Barley, 2001)

organisation and *as1* and *35S::BP* showing ectopic meristems and defects at the petiole/leaf blade boundary (Byrne *et al.*, 2000; Chuck *et al.*, 1996; Semiarti *et al.*, 2001; Sun *et al.*, 2002).

1.21 *BP* has a redundant role in SAM initiation and maintenance

knox gene redundancy in SAM initiation and maintenance in *Arabidopsis* was first suggested when plants expressing *BP* under control of the *35S::CaMV* promoter were shown to develop ectopic meristems on leaves (Chuck *et al.*, 1996; Lincoln *et al.*, 1994a). Electron micrograph analysis of *as1-magnifica* mutants also demonstrated formation of ectopic meristems which might be attributed to ectopic *knox* expression (Byrne *et al.*, 2000). Further evidence that *BP* acts redundantly with *STM* in the SAM was revealed in *stm*-2, *bp* double mutants. *stm-2* is a weak allele, that in contrast to strong *stm* mutations, does not prevent vegetative development. The activity of the SAM in *stm-2,* however, is dependent on BP activity because *stm-2 bp* double mutants show a stronger *stm* phenotype. The SAMs of double mutants abort following development of a small number of abnormal leaves. In support of a role for *BP* in the SAM, plants carrying the *as1-1*, *stm-1* and *bp* mutations lack a functional SAM despite the absence of both *AS1* and *STM* suggesting that *BP* is required to maintain the SAM in the absence of *STM* and without repression by *AS1*. The epistasis of strong *stm* mutations to *bp* is consistent with an *STM* acting earlier or upstream of *BP*, for example in specification of SAM cells in which *BP* is subsequently expressed (Byrne *et al.*, 2002).

1.22 The *serrate* mutation suppresses *as1* and *as2* mutant phenotypes, but increases the number of lobes

SERRATE is required maternally for normal embryo development. When self fertilised, *se* mutants often feature a single fused, or extra cotyledons (Prigge and Wagner, 2001). During vegetative development, fewer juvenile leaves but more adult leaves are formed. The first two leaves of *se* mutants show the complex venation and five hydathodes characteristic of the third leaf of wild-type (Clark *et al.*, 1999). All leaves have pronounced serrations at the margins, with the tip of each corresponding to a hydathode. The spiral phylotaxy of wild-type leaves results from initiation of leaves at an angle of about 137° following simultaneous initiation of the first two leaves at opposite sides of the SAM, and initiation of the third leaf at a position closer to one of the first two primordia (Callos and Medford, 1994). In *serrate* mutants, the first two leaves are initiated at an angle of approximately 137°, consistent with later leaves of wild-type. Subsequent leaves are initiated at a slower rate than wild-type, and at an approximate angle of 90° resulting in regular phylotaxy. *se* mutants bolt at the same time as wild-type, but in short day conditions produce aerial rosettes (Clark *et al.*, 1999), a feature of weak *stm* and *as1 stm* double mutants in which it occurs independently of day length (Byrne *et al.*, 2000).

Meristem structure is also altered in *se* mutants. The height of the SAM is increased resulting in meristem zones of larger size. As a result, the areas of

STM and *BP* become larger, but remain within their normal zones of expression (Ori *et al.*, 2000).

Double mutants between *as1 se* or *as2 se* have deep lobes that are also characteristic of *35S::BP* leaves. The double mutants have a larger number of lobes than *as1* or *as2* mutants and the lobes occur throughout the leaf rather than just at the end close to the stem as in *as1* or *as2* single mutants. Ori, *et al.* (2000) interpreted this as a quantitative and qualitative enhancement of the *as1* lobeing phenotype. Other interpretations are, however, possible. *se* single mutant leaves have a larger number of serrations than wild-type. In wild-type leaves, serrations only appear at the end proximal to the stem, in the region where lobeing in *as1* occurs. In the *as1 se* double mutants, serrations extend further along the length of the lamina. The size of the serrations is not increased, as sinuses of lobes in *as1* mutants can run as deep as the midvein. Because *as1 se* double mutants have longer leaves and more pronounced petioles than *as1* single mutants *se* can be considered a suppresser of some characteristics of the *as1* phenotype. The increased leaf length of *as1 se* relative to *as1* can also account for an increased number of serrations. While *as1* and *as2* mutants normally mis-express *BP* and *KNAT2* in leaves, *se* mutant do not, except in the sinuses of leaf lobes (Ori *et al.*, 2000), suggesting that the suppression of leaf phenotypes by *se* might result from reduced *knox* mis-expression.

1.23 *SERRATE* encodes a Zn-finger protein

SE encodes a C_2H_2-type zinc-finger with a nuclear localisation signal. The C_2H_2-type zinc-finger is a motif shared with the FERTILISATION-INDEPENDENT SEED2 protein of Arabidopsis, which is required in complex with FERTILISATION-INDEPENDENT ENDOSPERM and MEDEA for repressive chromatin structure during gametophyte development (Spillane *et al.*, 2000). The Zn-finger domain is implicated in interaction of the complex with DNA.

In the embryo, *SE* is expressed in the shoot and root meristems and adaxially in torpedo stage cotyledons. Expression fades through remaining embryo stages so that mRNA is undetectable in the mature embryo. Meristem expression persists through vegetative, inflorescence and floral development. Adaxial *SE* expression is also a feature of leaf initials, although expression becomes patchy by the time the organs reach maturity. The lateral organs of the floral meristem also express *SE* in early development but expression does not persist in mature organs (Prigge and Wagner, 2001).

The *se* mutation occurs in the penultimate intron, and only one *se* allele has been found, leading to speculation that a strong *se* allele would be lethal. Transgenic plants with *35S::SE* constructs that do not complement the *se* mutation, and are therefore thought to cause co-suppression, show more extreme phenotypes including adaxially curled lamina, reduced inflorescence internode elongation and phylotaxy defects. The strongest co-suppression results in SAM arrest before leaf production, or following development of the

first or second leaf. The plants then develop a new meristem which goes on to produce leaves with serrated edges. Flowers are few and lack organisation. Variable organ number and radially symmetrical filaments are a further characteristic (Prigge and Wagner, 2001).

1.24 RNA-induced gene silencing has developmental roles in and around the shoot apex

Silencing of genes by antisense RNA is a mechanism present in lower eukaryotes, fungi, animals and plants for negatively regulating gene expression. It is known by different names in the different kingdoms but is often referred to in plants as post transcriptional gene silencing (PTGS). It features production of small, 21-26 nucleotide, RNAs that act as specificity determinants for down-regulating gene expression (Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000). Small RNAs are generated by cleavage of double stranded RNA in an ATP dependant reaction (Nykanen *et al.*, 2001; Tang *et al.*, 2003) by a family of proteins called Dicer which contain RNA helicase, dsRNA binding and ribonuclease III domains (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Ketting *et al.*, 2001). One or more members of the ARGONAUTE (AGO) protein family of unknown biochemical function are an essential requirement for PTGS. AGO proteins are thought to recruit the small antisense RNAs created by Dicer into a complex called RISC (RNA-induced silencing complex), which recognises and cleaves target RNAs at sites complementary to the small RNA (Elbashir *et al.*, 2001; Hammond *et al.*,

2001; Hutvagner and Zamore, 2002). RNA-dependent RNA polymerases can create further dsRNA for cleavage (Catalanotto *et al.*, 2000; Tang *et al.*, 2003). In plants, RNA-dependent RNA polymerases can create dsRNA for cleavage in several ways. In defence, it can act without the need for an endogenous primer when unusually high levels of a single stranded RNA species is present (Tang *et al.*, 2003), as would be found in viral attack. This is also a possible mechanism for transgene silencing and co-suppression; (Ketting and Plasterk, 2000). In plant development however, generation of microRNAs (miRNAs) produced by cleavage of stem-loop precursor RNA transcripts by Dicer is thought to regulate endogenous mRNA expression (Lagos-Quintana *et al.*, 2001; Reinhart *et al.*, 2002). Interestingly, CARPEL FACTORY (CAF), one of at least four Dicer proteins in *Arabidopsis* (Park *et al.*, 2002), and PINHEAD (PNH, also known as ZWILLE), an AGO family member (Moussian *et al.*, 1998) are not involved in silencing of transgenes (Morel *et al.*, 2002; Park *et al.*, 2002), CARPEL FACTORY does however cleave miRNAs (Reinhart *et al.*, 2002), suggesting separate PTGS machinery in plant defence and development. Interestingly, PNH has a 123 residue N-terminal domain that is absent from all other known AGO family proteins (Moussian *et al.*, 1998), and which might therefore have a development-specific role.

Mutations at the *PNH* locus are fairly pleiotropic. Embryos have defects in suspensor development. Flowers of *pnh* mutants have abnormal organ

numbers and leaves are narrower than wild-type and curl adaxially (McConnell and Barton, 1995).

Early in development, *PNH* transcript is present throughout four cell stage embryos and at the top of the suspensor but during embryo development, transcript is progressively confined to central areas, and to the adaxial side of the cotyledons so that ultimately in mature embryos *PNH* mRNA is found in provascular cells, the SAM and adaxially in cotyledons. In vegetative development, *PNH* expression persists in the SAM, developing vascular strands and in adaxial domains of the leaf (Lynn *et al.*, 1999; Moussian *et al.*, 1998). This pattern of expression has led to some authors to describe *PNH* as a dorsalising factor (Bowman, 2000; Lynn *et al.*, 1999). Of particular interest in this study however is the lack of a functional SAM in *pnh* mutants where, at the transition from embryo to vegetative development, a determinate structure is formed at the shoot apex in place of the SAM. This structure is generally a thin, radially symmetrical pin-like structure, a flat apex, or a leaf (McConnell and Barton, 1995; Moussian *et al.*, 1998). Detailed studies of earlier developmental stages suggest that a SAM is formed in *pnh* embryos. A histologically discernible SAM is present in torpedo stage embryos and this shows expression of *STM* (Moussian *et al.*, 1998). By walking stick stage cells at the apex appear to have undergone differentiation and no longer express *STM*. *pnh* mutants eventually form adventitious meristems in the axils of the cotyledons approximately 14 days after germination and these SAMs can go on to form a rosette that bolts and flowers, indicating that there

is not an absolute requirement for PNH activity in the SAM. Development of axilliary meristems is also effected in *pnh* mutants which produce determinate structures in leaf axils (McConnell and Barton, 1995). Further evidence that *PNH* promotes of stem cell fates is provided by the ectopic SAMs that form when it is driven from the *35S* promoter. Ectopic expression of *STM* and *CUC2* is associated with the ectopic meristems (Newman *et al.*, 2002).

The *ARGONAUTE1* gene of *Arabidopsis* also has developmental roles in addition to its other roles related to PTGS. It is expressed ubiquitously, and in its absence pleiotropic defects occur. Development of *ago1-1* mutant embryos appears normal except that seedlings have narrow cotyledons. Rosette leaves are very narrow and have extra mesophyll layers and show no obvious difference between petiole and leaf blade. Once the transition to flowering is made, a single shoot is formed which develops a terminal inflorescence. Filamentous structures are produced in place of cauline leaves, suggesting a lack of adaxial/abaxial differentiation, and no axilliary meristems develop at the base of these structures. The inflorescence stem is also thicker and often fasciated. In flowers, sepals and petals are narrow and pointed. Stamens lack anthers, and the two carpels do not fuse to form a normal pistil.

When *pnh* mutants are incorporated into an *arganoute* (*ago*) mutant background, embryos fail to develop bilateral symmetry. Suspensor cells divide inappropriately leading to multiple cell layers. Despite *STM* protein accumulation in both *ago* and *pnh* single mutants, there is no accumulation in double mutant embryos. Seed do, however, germinate after several weeks

delay. The shoot system in these plants is completely disorganised and filamentous organs form in an unpredictable arrangement. Vasculature is not arranged in continuous strands, and vascular cells are misshapen. The apical/basal axis is maintained as are the three cell layers, but *PNH* and *AGO* clearly act redundantly in important aspects of embryogenesis (Lynn *et al.*, 1999).

Both *pnh* and *ago* mutants have features of loss of adaxial/abaxial polarity, which together with the polar expression of *PNH*, suggests a role for PTGS in organ polarity. Key promoters of adaxial cell fate in Arabidopsis leaves, the parologous *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) genes, are thought to be negatively regulated by PTGS. *PHB* and *PHV* RNA is normally confined to adaxial organ primordial, presumably by PTGS repression in abaxial regions (Rhoades *et al.*, 2002; Tang *et al.*, 2003). Dominant mutations in *PHB* and *PHV* result in lateral organs that are radially symmetrical and consist of adaxial cell types. In addition, *PHB* and *PHV* are expressed ectopically in abaxial cells (McConnell and Barton, 1998; McConnell *et al.*, 2001). All dominant *phb* and *phv* alleles involve mutations in a region that encodes a potential ligand binding site. (McConnell *et al.*, 2001). Because these mutations cause ectopic abaxial expression, it is possible that they mimic the effects of binding an activating, adaxial ligand and that the ectopically active proteins promote ectopic accumulation of their own RNAs. However, the sequence of several microRNAs, including miR165, is complementary to the region disrupted in dominant *phb* or *phv* mutations.

This has lead to the suggestion that *PHB* and *PHV* transcripts might be destabilised by miRNA abaxially (Rhoades *et al.*, 2002). miR165 has been shown to be associated with a RISC, and furthermore cleavage of mutant *phb* and *phv* has been shown to be greater than 14-fold slower than wild-type, suggesting that PTGS is critical in removing adaxial signalling from abaxial tissues in leaves (Tang *et al.*, 2003).

1.25 Mechanisms controlling the transition from indeterminate to determinate cell fates remain poorly understood

A mechanism is beginning to emerge whereby SAM cells are maintained in a pluripotent state by *STM*, which in turn requires either directly or indirectly other factors such as the *CUC* genes and *PNH* to maintain its active state (Aida *et al.*, 1999; Long *et al.*, 1996; Lynn *et al.*, 1999; Moussian *et al.*, 1998). At the periphery of the meristem it is, however, negatively regulated by *AS1*, which is expressed solely in lateral organs. This mechanism reflects changes observed as meristematic cells undergo alterations in gene expression profiles, allowing differentiation and elaboration of organs. One of the roles of *AS1* in lateral organ development is negative regulation of *knox* expression and therefore meristem fate, but there is evidence to suggest that this is not the only function of *AS1*. *as1 bp* double mutants have an additive phenotype despite the reduction of ectopic knox expression in lateral organs due to defective *BP*. This is also the case with *as1 knat2* mutants (Byrne *et al.*, 2000; Byrne *et al.*, 2002). If *knox* repression was the only function of *AS1*, *bp* and

knat2 mutants would be expected to be suppressers of *as1* and this is not the case. This study describes the genetic identification of a potentially important target of *AS1*, *SYMMETRICA* (*SYM*). The *sym* mutation is a complete suppresser of the *as1* mutant phenotype. Furthermore, a genetic interaction with *pnh*, suggests further links with PTGS in transition of cells from pluripotent to determinate cell fates.

2.0 MATERIALS AND METHODS

2.1 GENERAL MOLECULAR BIOLOGY

2.1.1 Preparation of total *Arabidopsis thaliana* DNA

About 0.5 g of leaves were subjected to a CTAB extraction of total DNA. CTAB extraction buffer (5 ml) containing 100 mM Tris-HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB and 0.2% 2-mercaptoethanol (Rogers and Bendich, 1985) was heated to 60°C. Leaves, which had previously been pulverised under liquid nitrogen, were added and the mixture incubated at 60°C for 20-30 minutes. Protein and CTAB were extracted by addition of 5 ml of chloroform. The organic phase was separated by centrifugation (2000 x *g* for 15 min). The top (aqueous) layer was removed and the DNA precipitated by addition 2/3 volume of isopropanol. DNA was collected by centrifugation (2000 x *g*, 15 min), washed in 70% (v/v) ethanol, left to air dry and dissolved in TE buffer (10 mM Tris·Cl pH 8 and 1 mM EDTA pH 8).

2.1.2 Preparation of plasmid DNA from bacterial cultures

Plasmid DNA was prepared from bacterial cultures using a QIAprep Miniprep Kit (Qiagen). Centrifugation (11,000 x *g*, 2 min) of overnight bacterial culture (2 ml) was used to pellet bacteria before lysis and DNA purification as described in the manufacturer's instructions. DNA was eluted from spincolumns in a volume of 50 μl distilled H_2O.

2.1.3 Estimation of DNA concentration

Concentrations of DNA recovered from purification procedures were estimated by diluting 1:100 with distilled water before measuring absorbance in a UV spectrophotometer at 260 nm. An OD of 1.0 was considered to correspond to approximately 50 μg/ml.

2.1.4 Ethanol precipitation of DNA

The DNA solution was combined with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol before precipitation (-70°C, 10 min). Centrifugation (11,000 x *g*, 25 min) followed and the supernatant was discarded. Pellets were washed with 70% (v/v) ethanol and air dried. DNA was dissolved in an appropriate volume of TE or H_2O.

2.1.5 Phenol/chloroform extraction

Phenol, presaturated with 10 mM Tris-HCl pH 8.0, was mixed with an equal volume of chloroform and centrifuged (3,000 x *g*, 5 min) to separate organic and aqueous layers. DNA solutions were combined with an equal volume of phenol/chloroform, vortexed and centrifuged (11,000 x *g*, 25 min) to extract proteins. The upper, aqueous, layer containing the DNA was removed to a clean tube and the DNA recovered by precipitation (Section 2.1.4).

2.1.6 Restriction endonuclease digestion of DNA

Between 1 and 5 μg of DNA were digested in 10 μl reactions according to instructions supplied with the enzyme. Typical reactions contained 1.0 μl of 10 x buffer (supplied by the enzyme manufacturer), 0.5 μl of restriction endonuclease (~6 units). The digest was incubated for a minimum of 1 hour at the temperature advised by the enzyme manufacturer.

2.1.7 Polymerase chain reaction (PCR)

PCR buffer (50 mM Tris-HCl pH 8.3, 0.25 mg/ml crystalline BSA, 3 mM $MgCl_2$, 0.5% Ficoll 400 and 1 mM tartrazine), 200 μM of each dNTP (deoxynucleoside 5' triphosphate), 0.2 μM of forward and reverse primers and 0.4 units of *Taq* DNA polymerase (Roche Biochemicals) were combined in a total volume of 20 μl with, in typical reactions, 100 ng of genomic DNA template or 100 fg of plasmid template. The reaction was predenatured at 94°C for 2 min followed by 35 cycles of three temperatures: denaturation (94°C, 30 sec), annealing (between 45°C and 60°C, 30 sec) and extension (72°C) with an extension time of 1 sec/50 bp for products < 500 bp, or 1 sec/ 25 bp for products > 2kbp. A final extension at 72°C for 5 min was performed. Annealing temperatures were calculated according to primer melting temperatures and in some cases required optimisation for specificity (Saiki *et al.*, 1988).

Products were visualised on agarose gels. Purification of PCR reactions, when required, were carried out using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

PCR reactions were performed on a Rapidcycler (Idaho Technology) or a PTC-200 thermal cycler (MJ Research) The Rapid Cycler confers heat by hot air to samples sealed inside thin walled glass capillary tubes while the PTC-200 heats and cools samples in plastic tubes in contact with a plate.

2.1.8 Oligonucleotides

Oligo Name	Sequence (5' to 3')	Purpose
knat1-f knat1-r	catagatgagtcgtctagtcg catgtcacagtatgcttccatg	Testing *BREVIPEDICELLUS* expression by RT-PCR
knat2-f knat2-r	ggatagaatgtgtggtttccg gctgtagcagacgctggac	Testing *KNAT2* expression by RT-PCR
knat6-f knat6-r	caagcttacatcgattgcc cctggcagactcgacaccag	Testing *KNAT6* expression by RT-PCR
ACTIN2-f ACTIN2-r	tgtattgaactcttttt caacatatacataaataa	RT-PCR control
se or SE f se or SE r	ttgtaccagcaccacctgaa gtcacttcttcctctggagc	Genotyping plants for the *serrate* mutation.
35S en upper 35S en lower	gtaaaacgacggccagt acccgccaatatatcctg	Making southern probe for pSKI015 insert screens.
BIL 1f BIL 1r	ggtggtgctactactggtga tagtagttgtgtagccgtta	Testing *PETAL LOSS* expression by RT-PCR.
stm-1 cut2f STM-R 2160	tatgaacaagaatttgtagatg ttaaaaacatgaaatgattattt	Genotyping plants for the *shoot meristemless-1* mutation.

as1mutantcut-F	ttcagcctcctaacccagtg	Sequencing primers for *as1-1* mutant genotyping.
R4	gcgtctcgccttaaaccaac	
nga168f	gaggacatgtataggagcctcg	Mapping
nga168r	tcgtctactgcactgccg	
CER449030 F13M22f	ataaccacagatgcacaagc	Mapping
CER449030 F13M22r	ttcacttctcggcgctggcc	
CER452282 F4I1f	tagctagctggtagaagact	Mapping
CER452282 F4I1r	cctgatttgggtctccagat	
ciw4f	gttcattaaacttgcgtgtgt	Mapping
ciw4r	tacggtcagattgagtgattc	
nga692f	agcgtttagctcaaccctagg	Mapping
nga692r	tttagagagagagagcgcgg	
nga1126f	gcacagtccaagtcacaacc	Mapping
nga1126r	cgctacgcttttcggtaaag	
nga248f	tctgtatctcggtgaattctcc	Mapping
nga248r	taccgaaccaaaacacaaagg	
MBK-5f	gagcatttcacagagacg	Mapping
MBK-5r	atcactgttgtttaccatta	
nga139f	agagctaccagatccgatgg	Mapping
nga139r	ggtttcgtttcactatccagg	
CER449255 F14M4f	ggtcattatggtctgggctt	Mapping
CER449255 F14M4r	catcatatcatgtgactggt	
CER452337 F4L23f	ctgatatgtagaacgcaatc	Mapping
CER452337 F4L23r	aatccatcatagcatcgggc	

CER460491 T3F17f	cgattctcaaagtcttatct	Mapping
CER460491 T3F17r	cacttgcgcgtctaatccaa	
CER452340 F4I18f	catgacgttaccatatactt	Mapping
CER452340 F4I18r	gtctagctatcttgtcctaa	
CER449811 F17K2f	cattacatgttggagttttg	Mapping
CER449811 F17K2r	tgttatgacaaggccatgcc	
CER448603 F11C10f	atggcaagaaatgaactcca	Mapping
CER448603 F11C10r	ccagctatttgacaattacc	
CER446344 T3A4f	gcttgagaactttcttcact	Mapping
CER446344 T3A4r	agcgaaaacgtggtatacgt	
CER460253 T30B22f	gcctatgctagtctccagac	Mapping
CER460253 T30B22r	gaattacatggttatggcca	
fus6.2f	ttccttgatcagatttggtcg	Mapping
fus6.2r	tcgttacactggcttgcttg	
SKI015 RI	gcaagaacggaatgcgcg	Sequencing from rescued activation tagging plasmids.
NAM f	gagcatatcagtgaacggaca	RT-PCR on At2g46770.
NAM r	tctttccctccatttccgtt	

2.1.9 Gel electrophoresis of DNA

Small amounts (< 0.5 μg) of DNA were resolved on meniscus gels poured on glass plates (15 cm x 6 cm) underlying a comb of required size to form wells. 0.3 g of agarose in were heated in 30 ml 0.5 x TBE until dissolved. Ethidium bromide solution was added to 10 μg/ml for subsequent visualisation prior to gel casting. Gels were set and equilibrated in an electrophoresis gel tank with 0.5 x TBE as tank buffer. DNA samples were mixed with 0.1 volume of loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% (w/v) Ficoll type 400) prior to loading the gel. DNA was subjected to a potential gradient of 10 V/cm until the bromophenol blue dye front was approximately half way down the gel. DNA was visualised on a UV transilluminator (UVT-28MP, E.A.S.Y., Herolab).

2.1.10 Mapping of a mutation relative to molecular markers

One commonly used approach to determine a relative map position for a mutation is to generate an F2 population by crossing the mutant with a wild-type plant from a different ecotype. DNA is then extracted from F2 plants with the genotype which can be unambiguously deduced from the phenotype (i.e. homozygous mutants, if the mutation is recessive). Molecular markers that are polymorphic between the two parents and located throughout the genome are then used to test for co-segregation of a molecular marker with the mutant phenotype. This method had to be adapted for mapping the *sym*

mutation, for three reasons. (1) *sym* conditioned no mutant phenotype in an *AS1*$^+$ wild-type background, therefore *as1* mutants had to be used as the *SYM*$^+$ mapping parents. (2) The *sym* mutation had been generated in an *as1-1* mutant that proved to have a mixed genetic background from Col and L*er* ecotypes. Therefore mapping populations were generated from crosses to available mutations *as1* mutations in Col-0, L*er* or En backgrounds. (3) *sym* proved to be semi-dominant in these mapping crosses and *sym/sym* homozygotes with a wild-type phenotype could not be distinguished unambiguously from *sym/SYM*$^+$ heterozygotes with a partially suppressed *as1* mutant phenotype. Therefore plants (n = 30 or 96) with the strong *as1* mutant phenotype, assumed to be *SYM*$^+$/*SYM*$^+$ homozygotes, were used for genotype analysis. The markers used were mostly simple sequence length polymorphisms (SSLP's) which take advantage of insertions or deletions of DNA that distinguish different ecotypes (Bell and Ecker, 1994). PCR using oligonucleotides flanking these SSLP's (Section 3.1.8) and electrophoresis of PCR products on 3% agarose gels allowed alleles to be distinguished as differences in fragment length. If *SYM* was unlinked to a molecular marker, 25% of *SYM*$^+$/*SYM*$^+$ homozygotes were expected to be homozygous for one allele (visible on the agarose gels by a single band), 25% homozygous for the other allele (visible as a single band of different size) and 50% heterozygous (visible as both bands, often with additional bands representing heteroduplex DNA). The numbers of each genotype were counted and the chi-squared test used to test whether the observed proportions differed significantly from that

expected using the formula $\chi^2 = \Sigma (o - e)^2 / e$, where *o* is the observed number of plants with a specific genotype, and *e* is the expected number of plants with that genotype if the marker and *SYM* are unlinked. The significance of the chi-squared value was then calculated online (http://www.physics.csbsju.edu/stats/chi-square_form.html). Significantly different segregation (i.e. fewer than expected alleles from *as1-1*) indicated that *SYM* was linked to the marker. Linkage to marker *nga361*, allowed *SYM* to be assigned to the lower end of Chromosome 2.

2.1.11 Using a large F2 population to fine-map a mutation

The ability to fine map a mutation depends on the number of recombination events separating the mutation from flanking markers. Therefore larger F2 mapping populations are required. F2 mapping populations were generated as described in the previous section. Tissue from 328 SYM^+/SYM^+ homozygotes was collected and subjected to DNA extraction. Plant material was ground wet in 0.5 M NaOH (10 μl per mg of tissue). An aliquot (5 μl) of this extract was then added to 495 μl of 100 mM Tris pH 8. This mixture was stored at -20°C and 2 μl of it used in each 15 μl PCR reaction with SSLP primers (Wang *et al.*, 1993). SSLP's in known positions were designed from comparisons of Col-0 and L*er* sequences in linked regions (http://www.arabidopsis.org). In the initial screen, 94 plants were tested for recombinations between the *SYM* and a linked marker. Recombinants in this interval were selected for further analysis. A marker to one side of the first was then used to map *SYM* to a

position either higher or lower than the first marker by comparing the number of recombinants for each. If fewer plants were recombinant at the second marker locus, then the *SYM* lay closer to the second marker. Plants with a recombination event between *SYM* and the nearer marker were then tested for recombination further along the chromosome using a different polymorphic marker. Non-recombinants were discarded, and the process repeated until a marker was found which was not associated with any recombination events between it and *SYM* in the 96 F2 plants. The larger population of 328 plants was then screened for further recombinants at this marker locus. If the marker was to the same side of *SYM* as the previous markers, then the plants that were recombinant for the nearest marker should also be recombinant for the others. If however, the nearest marker lay to the other side of *SYM*, then recombinants for the nearest marker were unlikely to be recombinant for the others. Using this method *SYM* was mapped to chromosome 2, south of marker *CER446344* and north of marker *CER449255*.

2.1.12 Isolation of DNA fragments from agarose gels

DNA bands in agarose gels were visualised on a UV transilluminator and excised with a scalpel. The gel fragment was weighed in an Eppendorf tube and the DNA purified using a QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions.

2.1.13 Dephosphorylation of vector ends

Alkaline phosphatase was used to catalyses dephosphorylation of either 5' protruding or 5' recessed ends following digestion of vector DNA. As ligation reactions require a phosphate group, dephosphorylation of the vector means that this phosphate must come from the insert DNA, preventing self-ligation of the vector.

One unit of Shrimp Alkaline Phosphatase (Roche Diagnostics) was added to 7 μl of digested vector DNA (< 1 μg) in 1 x manufacturer's buiffer, incubated (10 min, 37°C) and inactivated (65°C, 15 min). DNA was recovered by ethanol precipitation or using QIAquick columns.

2.1.14 Ligation of DNA

Ligation of DNA into vectors was carried out using a 3:1 molar ratio of insert DNA to vector DNA in 10 μl reactions containing 1.0 μl of 10 x Buffer (supplied by NEB) and 5 units of T4 DNA ligase (NEB). Reactions were incubated at 15°C for 1 hour, or 4°C overnight.

Ligations into the pGEM-T vector (Promega) were performed according to the manufacturer's instructions.

2.1.15 Preparation of competent *E. coli* for heat-shock transformation

An aliquot of overnight culture (1.5 ml) of DH10B was added to 50 ml of pre-warmed L-broth and left on a shaker at 37°C for 2 hours. The culture was transferred to 50 ml Falcon tubes and centrifuged at 5,000 x *g* at 4°C for 5 min. The pellet of bacteria was resuspended in 4 ml of ice cold 100 mM $MgCl_2$ and chilled on ice for 30 min. Cells were spun down as before, resuspended in 4 ml 100 mM $CaCl_2$, centrifuged again before resuspension in 2 ml $CaCl_2$ solution and incubated on ice for 1 hour.

2.1.16 Transformation of *E. coli* by heat shock

For each transformation, a 100 μl aliquot of competent cells were mixed with approximately 10 ng of plasmid DNA and incubated on ice for 30 min. Cells were subjected to heat-shock at 42°C for 2 min and transferred back to ice for several minutes. L-broth (1 ml) was added to the cells which were then incubated at 37°C for 1 hour. Cell suspensions were spread on LB agar plates containing antibiotic for selection of transformants and incubated at 37°C overnight.

2.1.17 Preparing electro-competent *E. coli*

An aliquot of 10 ml of a DH10B (F-*mrc*A δ (*mrr-hsd*RMS-*mcr*BC) φ80d*lacZ* δM15 δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 δ (*ara,leu*) 7697 *gal*U *gal*K λ- *rps*L *nup*G) overnight culture was added to 500 ml of L-broth and left on a shaker at 18°C until an OD_{600} of 0.4 was reached. The cells were collected by centrifugation for 15 min at 5,000 x *g* at 0°C and washed in 500 ml ice-cold sterile water. Washes were repeated with 250 ml sterile ice-cold water and 20 ml sterile ice cold water, with cell collections by centrifugation as before. Once the cells were in a 20 ml suspension, a 5,000 x *g* centrifugation at 0°C in swingout rotor was used to collect the cells in a small pellet for resuspension in 0.8 ml of ice-cold 0.7% DMSO. Aliquots of 0.1 ml were snap-frozen and stored at -70°C until required.

2.1.18 Electro-transformation of *E. coli*

An aliquot of 1 - 2 μl containing ~10 ng of plasmid DNA was combined with 40 μl of cells previously thawed on ice. The mixture was transferred to chilled electroporation cuvettes (1 mm gap) and incubated on ice for 30 min. The cells and DNA were and pulsed in the cuvettes at 50 μF 1800 V, 150 Ω before addition of 800 μl of SOC medium and incubation at 37°C with shaking for 1 hour. Transformed bacteria were recovered as in 2.1.16.

2.1.19 DNA sequencing

PCR products purified from agarose gels using a QIAquick Gel Extraction Kit were used as DNA template for sequencing. Templates (~90 ng of PCR product) were combined with 3.2 pmol of sequencing primer and 8 μl of ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction mix (containing AmpliTaq DNA Polymerase, dye terminators, dNTP's, magnesium chloride and buffer) in 20 μl reactions.

Sequencing reactions were subjected to 26 cycles of 96°C (30 seconds), 50°C (15 seconds), and 60°C (4 minutes) in the PTC-200 thermal cycler. Extension products were purified by addition of sequencing reactions to tubes containing 2 μl of 3M NaOAc pH 4.6 and 50 μl of 100% ethanol. Tubes were vortexed and incubated at -20°C for 10 min. Reaction products were collected at 11,000 x *g* and washed with 250 μl of 70% ethanol and dried in a vacuum centrifuge for approximately 5 min. Samples were sent to the University of Edinburgh, ICMB sequencing unit and subjected to analysis on an ABI 3100 Genetic Analyzer. Returned sequencing files were analysed using computer programmes DNAstar and BLAST.

2.1.20 Freeze-thaw transformation of *Agrobacterium tumefaciens*

Agrobacterium strain GV3101 carrying the MP90 Ti plasmid was grown in 100 ml LB with 50 μg/ml gentamicin shaken at 28°C until an $O.D._{680}$ of 0.5-1.0 was reached. Cultures were chilled on ice and cells were collected by

centrifugation as for *E. coli.* Supernatant was discarded and cells resuspended in 5 ml ice-cold $CaCl_2$ (20 mM). Aliquots of 0.2 ml were transferred to pre-chilled sterile microcentrifuge tubes. Approximately 10 μg of plasmid DNA in 5-10 μl of sterile H_2O were added to cell suspensions before incubation on ice for 30 min. Cell suspensions with DNA were frozen in liquid N_2 followed by incubation at 37°C for 5 min. One ml of LB was added prior to incubation with gentle shaking at 28°C for 2 hours. Cells were pelleted by brief centrifugation, the supernatant discarded and the cells plated on LB agar plates containing 100 μg/ml rifampicin (to select for the bacterial chromosome), 50 μg/ml gentamicin (for Ti plasmid selection), and 50 μg/ml kanamycin for binary plasmid selection. Plates were incubated for 2 days at 28°C.

2.1.21 Southern blotting

Approximately 10 μg of genomic DNA was subjected to restriction enzyme digest as described in Section 3.1.6, except that spermidine sulphate was added to a final concentration of 1 mM, and digests were left for 4 hours. Digested DNA was separated in 0.7% agarose gels (Section 3.1.9). No ethidium bromide was added to the agarose, but gels were stained in 1 μg/ml ethidium bromide in 0.5x TBE for photography following electrophoresis.

Gels were prepared for blotting onto nylon by 10 min agitation in 0.25 M HCl to nick DNA and allow transfer of large DNA fragments. Gels were then rinsed in water prior to successive agitations in denaturing solution (1.5 M

NaCl, 0.5 M NaOH) followed by neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) for 20 minutes each.

The gel was placed on Saranwrap. Hybond-N nylon filter (0.45 μm pore size, Amersham) was cut to size, pre-soaked in neutralising solution and placed flat on the gel. Whatmann 3MM paper and paper towels were stacked on top of the filter and the whole apparatus was gently pressed by a flat heavy object rested on top. The gel was left overnight to allow transfer of DNA to the nylon filter.

Following transfer of DNA, the filter was removed and soaked briefly in 2 x SSC (100 mM NaCl, 10 mM Na citrate) and air dried. DNA was crosslinked to the filter using 0.4 J cm^{-2} of UV light in a transilluminator.

2.1.22 Oligo-labelling of DNA

Probes were made by PCR in 50 μl reactions as described previously, except that a mix containing digoxygenin-labelled dUTP (Roche 10x labelling mix) was used in place of unlabelled dNTPs.

2.1.23 Hybridisation

Nylon filters were pre-hybridised for 2 hours at 65°C in 70 ml hybridisation buffer (1 % (w/v) skimmed milk powder, 1% (w/v) SDS, 5 x SSC, 1 % N-lauryl sarcosine). Probes (20 μl of the PCR reaction) were denatured at 100°C for 5 min and combined with 20 ml hybridisation buffer prewarmed to 65°C.

2.1.24 Washing probed Filters

For removal of background signal, filters were twice agitated for 5 min at 25°C in 100 ml 2 x SSC, 0.1 % (w/v) SDS, before being washed twice at high stringency (68°C, 15 min) in 100 ml 0.5 x SSC, 0.1 % (w/v) SDS.

2.1.25 Visualisation of target DNA

Filters were agitated for 30 min at 25°C in 70 ml blocking buffer (1% (w/v) Roche blocking reagent, 100 mM maleic acid, 150 mM NaCl pH 7.5) prior to incubation for 30 min at 25°C with 2 μl alkaline phosphatase-coupled anti-DIG antibody (Roche, FAP fragments) in 20 ml blocking buffer. Two 15 min washes in 0.3% Tween 20, 100 mM maleic acid, 150 mM NaCl pH 7.5 followed before equilibration of filters in detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl) for 3 min and exposure to chemiluminescent substrate solution (Roche CSPD, diluted 1:50 in detection buffer). Filters were sealed in bags, following exclusion of air bubbles, and exposed to X-ray film. Exposure times varied according to the strength of signal. X-ray films were developed using an automatic developing unit.

2.1.26 Preparation of total RNA from *Arabidopsis thaliana*

Approximately 100 mg of plant tissue were ground with glass beads in 1 ml of Tri-reagent (Sigma) using a pestle and mortar. Following transfer to a 1.5 ml Eppendorf tube, samples were allowed to stand for 5 min before addition of 0.2 ml of chloroform. Tubes were vortexed and left at room temperature for 10 min before centrifugation at 11,000 x *g* at 4°C for 15 min. The aqueous

(upper) phase was removed and added to a fresh tube containing 0.5 ml isopropanol. Samples were left at room temperature for 10 min to allow complete precipitation of RNA prior to centrifugation (as before) to collect RNA. The supernatant was discarded and the pellet washed in 1 ml of 75% ethanol before collection by centrifugation at 11,000 x *g* at 4°C for 5 min. Pellets were air dried and dissolved in distilled, autoclaved water.

2.1.27 Production of cDNA *Arabidopsis thaliana* RNA

Approximately 5 μg of total RNA in 20 μl of water was heated at 80°C for ~ 3 min. Superscript II reverse transcriptase buffer (4 μl), dNTPs (0.5 mM each), DTT (10 mM), QT poly-T primer (0.4 μM; Frohman, *et al.*, 1988), RNAsein (0.5 units) and Superscript II Reverse Transcriptase (10 units, NBL) was added. The mixture was placed in a PTC-200 thermal cycler (MJ Research) and subjected to heating at 42°C for 1 hour, 50°C for 10 min and 70°C for 10 minutes. cDNA was diluted to 100 μl with water and 2 μl used as PCR template.

2.1.28 Plasmid rescue of pSKI015 T-DNA from *Arabidopsis* DNA

Plasmid rescue was used to recover sequences adjacent to T-DNA insertions in *Arabidopsis* plants by making use of the pBluescript backbone present in pSKI015, which contains an ampicillin resistance gene and origin of replication (Weigel, *et al.*, 2000). Approximately 10 μg of genomic DNA

from the plant of interest was subjected to *Eco* RV digestion as in section 2.1.21. *Eco* RV cleaves once within the vector, and at sites in the adjacent plant DNA *Eco* RV was inactivated by phenol/chloroform extraction and DNA recovered by ethanol precipitation and dissolved in 10μl of sterile water. The DNA fragments were subjected to ligation in a large volume to encourage intra-molecular ligation by addition of 5.0 μl of 10 x ligation buffer (NEB), 2.5 μl of 20 mM spermidine sulphate, 25.5 μl of water and 25 units of T4 DNA ligase (NEB). Reactions were incubated at 4°C overnight. Recircularised vector was recovered by electro-transformation of *E. coli* and selection agar plates containing ampicillin. Overnight cultures were made from surviving bacterial colonies and plasmids purified as in Section 2.1.2. The plasmid, containing part of the original T-DNA and adjacent plant DNA, was subjected to sequencing from primers flanking the *Eco* RV site in the T-DNA. Determination of the site where the original pSKI015 plasmid was incorporated into the plant genome was then carried out by comparison of non-T-DNA sequences adjacent to the *Eco* RV site to the *Arabidopsis* genomic sequence using BLAST.

2.2 PLANT MATERIAL

2.2.1 *Arabidopsis thaliana* stock lines

The following lines were obtained from the *Arabidopsis* Biological Resource Center (ABRC), Ohio, USA:

Mutant	Allele	Background	Stock No.
asymmetric leaves1	*as1-1*	L*er*/ Col-2	CS146
asymmetric leaves1	*as1-mag*	En	CS3283

These lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC), UK:

Mutant	Allele	Background	Stock No.
asymmetric leaves1	*as1-1*	Col-1	N3374
asymmetric leaves2	*as2-2*	An	N3117
brevipedicellus	*bp-1*	L*er*	NW30
pinhead	*zll-3*	L*er*	N11
serrate	*se*	Col-1	N3257
shootmeristemless	*stm-1*	L*er*	N8154

The following lines were obtained from other sources:

Mutant	Allele	Background	Scource
asymmetric leaves1	*as1-12*	L*er*	Gift from Juan Micol, Univ. Miguel Hernandez.
p*35S::KNAT1*	n/a	Col	Gift from Sarah Hake, University of Berkeley.
p*KNAT1::GUS*	n/a	L*er*	Gift from Miltos Tsiantis, University of Oxford.
p*STM::GUS*	n/a	Col-0	Gift from Kathryn Barton, University of Wisconsin.

2.2.2 Plant growth conditions

Seeds were imbibed at 4°C for 3 - 4 days in 0.1% agarose and grown on Levington M3 potting compost. Plants were grown in growth rooms at an average temperature of 20°C, supplied with light from fluorescent tubes.

2.2.3 Methane sulphonic acid ethyl ester (EMS) mutagenesis of *as1-1* seed

EMS is a chemical mutagen that ethylates guanine and causes transitions from GC to AT generating mainly point mutations loss-of-function phenotypes. *as1-1* seeds (0.2 mg) were washed in 0.1% (v/v) Tween 20 for 15 min, before addition of 0.3% (v/v) EMS solution (15 ml) and placed on an end-over-end mixer for 10 hours. Following removal of the EMS solution, seeds were washed in 10 ml H_2O for 4 hours on the mixer. The procedure was carried out

in a fume hood. Seeds were imbibed and plants grown as in Section 2.2.2. M1 plants were grown in groups of five and allowed to set seeds. M2 seeds were collected from each family of five plants, sown and screened for mutant phenotypes.

2.2.4 Transformation of *Arabidopsis thaliana*

Large liquid cultures of *Agrobacterium* (400 ml per 9 cm pot of plants to be treated) were grown to an $O.D._{600}$ of 0.8. Cells were harvested by centrifugation (Section 3.1.20), and resuspended in 5% (w/v) sucrose solution of an equal volume to the original culture. Silwet L-77 was added to a final concentration of 0.05% (v/v). Above-ground parts of healthy, flowering plants were briefly dipped with gentle agitation in the *Agrobacterium* suspension. Plants were then covered for 24 hours, and seed harvested once the plants were mature and dry (Clough and Bent, 1998).

2.2.5 T-DNA activation tagging in *Arabidopsis*

The pSKI015 activation tagging vector features four *35S CaMV* enhancers, which can up-regulate genes adjacent to the T-DNA when it is inserted into the *Arabidopsis* genome. Other features are a BAR^R, a gene conferring resistance to the commercial herbicide BASTA for selection of transgenic plants, and a pBluescript backbone with an ampicillin resistance gene for selection in *E. coli* (Weigel *et al.*, 2000). The vector was obtained as a

glycerol stock in *E. coli* strain DH10B (a gift from Justin Goodrich) and plated out on LB agar with antibiotic selection (Section 3.1.20) and left at 37°C overnight. Cultures of vector-carrying bacteria from single colonies were incubated in L-broth with antibiotic selection overnight. Plasmid was recovered from bacterial cultures (Section 2.1.2) and used to transform *Agrobacteruim* (Section 2.1.20). Large cultures of vector carrying *Agrobacteruim* were grown which were used to transform *Arabidopsis* (Section 2.2.4) and T1 plants selected with BASTA and screened for novel phenotypes. T1 plants were also allowed to set self seeds and the T2 generation screened for loss-of-function mutations, in families from five T1 plants.

2.2.6 Genetic crosses

Flowers to be used as female (pollen acceptor) were emasculated before anthesis. Pollen was transferred from the male (pollen donor) using fine watchmakers forceps.

2.2.7 Vapour-phase sterilisation of *Arabidopsis* seed

Under a fume hood, microfuge tubes of seed were placed in a dessicator jar with a beaker containing 100 ml commercial bleach. Concentrated HCl (3 ml) was added to the bleach and the jar immediately sealed. Sterilisation by

chlorine gas was left to proceed for 8 hours. The jar was opened in a laminar flow hood and the tubes immediately sealed.

2.2.8 GUS staining of plant material

Plant tissue was vacuum infiltrated for 10 minutes under a solution containing 25 mM phosphate buffer, pH 7.0, 0.25% (v/v) Triton X-100, 1.25 mM potassium ferricyanide, 1.25 mM potassium ferrocyanide, 0.25 mM EDTA, 1 mg/ml X-Gluc (5-bromo-4-chloro-3indolyl-β-D-glucuronide), and incubated overnight at 37°C. Plant material was cleared for analysis of GUS expression in 70% ethanol.

3.0 GAIN OF FUNCTION MODIFIER SCREEN IN AN *as1* MUTANT BACKGROUND

3.1 RESULTS.

3.1.1 Gain-of-function mutagenesis in an *as1* mutant background.

A limitation of loss-of-function screens in wild-type backgrounds is that they rarely identify genes that act redundantly. Additionally, genes required for multiple stages of the plant life cycle often confer embryonic or gametophytic lethality making the difficult to identify as strong loss-of-function mutations.

Both redundant genes and genes essential for early survival can be identified by gain-of-function mutagenesis screens, for example by using the activation tagging vector pSKI015, in which via four copies of the cauliflower mosaic virus *35S* enhancer can drive strong expression from active promoters, resulting in loss of tight transcriptional regulation (Weigel *et al.*, 2000).

To apply this technique to identify genes interacting with *AS1*, 13,000 *as1-1* mutants were subjected to the floral dip method of plant transformation (Clough and Bent, 1998) using the pSKI015 activation tag T-DNA. T1 seeds were collected from pools of ~60 T0 plants and transformants were selected by germination on compost soaked with BASTA herbicide. 6000 BASTA resistant T1 plants were analysed. The main objective of the screen was to identify targets of *AS1* repression as dominant enhancers of the *as1* mutant phenotype and downstream targets of *AS1* required for exclusion of expression of meristem genes from lateral organs as dominant suppressers.

3.1.2 *35S$_{en}$::BIG PETAL* mutants develop large petals in the presence of the *as1-1* mutation

All lateral organs of *as1* mutants have developmental defects. Their petals are narrower and less regularly shaped than wild-type and occasionally reduced in number. The *35S$_{en}$::BIG PETAL* (*35S$_{en}$::BIL*) transformant was identified as having a suppressed *as1-1* petal phenotype in the M1 population, suggesting a dominant mutation resulting from up-regulation of the *BIL* gene. *BIL* was therefore considered a possible target of *AS1* that could be involved in exclusion of meristem gene expression from lateral organs. The vegetative phenotype of the *as1-1 35S$_{en}$::BIL* plant in the M1 population did not appear different from *as1-1* single mutants (Fig. 3.1; Fig. 3.6).

In order to determine a phenotype caused by upregulation of *BIL* in the absence of *as1-1*, *as1-1 35S$_{en}$::BIL* double mutants were crossed to Col-0. Rosette leaves of the F1 population were serrated at the margins and curled adaxially (Fig.. 3.6). Following the transition to floral development, the internode length was extremely variable with some nodes close together and others with normal separation (Fig 3.7). Other striking features were petals that were larger than wild-type and more numerous. Although most flowers developed four petals, flowers with five, six or seven petals were regularly observed.

The number of activation tagging vectors incorporated into the genome of the *35S$_{en}$::BIL* plant was unknown, so to ensure that all the observed phenotypes were caused by a single T-DNA insert the F2 population were sprayed with

Figure 3.1 *35Sen::BIG PETALS* floral phenotype.
a wild type flowers. **b** *as1-1* flowers **c** *as1-1 35Sen::BIL* flowers in the T1 population. *as1-1 35Sen::BIL* show suppression of the *as1-1* single mutant phenotype - large petals were developed without the wrinkled appearance of *as1* mutant petals. **d** F1 plant from *as1-1 35Sen::BIL* x Col-0. F1 plants develop flowers with increased petal number.

BASTA herbicide to screen for the presence of the transgene. Three quarters of the F2 population survived, suggesting that the T-DNA had inserted at a single locus and that all the observed phenotypes were likely to be the result of upregulation of a single gene, *BIL*.

3.1.3 The *35S*$_{en}$*::BIL* phenotype is caused by a double T-DNA insertion 5' of a GT-2 like transcription factor gene

Genetic evidence suggested that the novel phenotypes were the result of insertion at a single locus. This was tested further by Southern hybridisation. DNA was extracted from eight BASTA-resistant F2 plants with the *35S*$_{en}$*::BIL* phenotype, digested with *Eco R*I, which cuts at a single site in the activation tagging vector, separated by size and probed with the *35S* enhancer sequence. Each T-DNA insertion was expected to produce a different band with a size dependent on the proximity of the *Eco* RI flanking the T-DNA insertion. In all of the eight plants, two distinct bands corresponding to individual inserts were observed. This suggested that the inserts were linked, consistent with the 3:1 segregation of BASTA resistance in this generation.

In order to determine where in the plant genome the T-DNAs had inserted, fragments from the *Eco R*I digest were circularised by ligation at low concentration and used to transform *E. coli*. Bacteria that survived selection on ampicilin were assumed to carry a plasmid consisting of the pBluescipt backbone of pSKI015, additional T-DNA sequences and Arabidopsis DNA

flanking the insertion site. Since *Eco RI* cut once in the vector, plant sequences adjacent to vector sequence should have been present in the rescued plasmids. The rescued plasmids were therefore sequenced using an oligonucleotide complementary to a region internal to the *Eco RI* site in the T-DNA. Two sequences were obtained. Sequence from one of the rescued plasmids (Plasmid A) consisted of T-DNA sequence up to the *Eco RI* site (Fig.. 3.2), followed by additional T-DNA sequence, suggesting that two T-DNAs had inserted in tandem. The second sequence (Plasmid B) consisted of T-DNA sequence to the *Eco RI* site followed by Arabidopsis DNA from a position ~975 kb from the upper end of chromosome five. This Arabidopsis DNA consisted of part of an intron and exon of gene At5g03680 in BAC F17C15 (Fig. 3.3, 3.4). The gene had a single *Eco RI* site 1518 bases from the start of the At5g03680 ORF, corresponding to the site at which flanking DNA had been ligated to the internal *Eco RI* site of pSKI015. In order to determine an approximate location for the T-DNA insert, plasmid B was linearised by digestion with *Eco RI*, and digested with *Eco RV*, which cuts after the *35S* enhancers in the T-DNA. The resulting fragments were separated on an agarose gel. A band of approximately 4 kb was observed in the *Eco RV* digest, corresponding to the T-DNA. Two further bands of ~5 kb and 0.7 kb were also observed. An *Eco RV* site is present at 3,296 bp upstream of the beginning of the At5g03680 ORF and the distance from the transcription start site to the *Eco RI* site is 1541 bp. Therefore the ~5 kb band was consistent with the expected fragment of 4737 bp, suggesting that the site of T-DNA

insertion was ~4 kb from the start of the At5g03680 ORF (Fig 3.4). The size of the *Eco* RI fragment (insert size) was also consistent with this position. The double T-DNA insert therefore appeared to be located between genes At5g03670, which encodes a protein of unknown function, and At5g03680, which encodes a GT-2 like transcription factor. The *35S* enhancers were pointing towards At5g03680, making this gene the strongest candidate for *BIL*. At5g03680 was subsequently found to correspond to a gene previously identified by its loss-of-function phenotype as *PETAL LOSS* (*PTL*; Philip Brewer, personal communication), a gene required for correct orientation and maintenance of second whorl organs (Griffith *et al.*, 1999). The *BIL* gene was therefore renamed *PTL*.

In order to confirm that upregulation of *PTL* was responsible for the mutant phenotypes observed, the levels of *PTL* expression were tested by RT-PCR. primers were used that would amplify a *PTL* fragment of 1499 bp from genomic DNA template, or 373 bp from cDNA template. In both wild-type and in *as1-1* mutant leaves, *PTL* expression was below the level of detection. In $35S_{en}$*::PTL* mutants, expression was clearly detected (Fig 3.5), suggesting that *PTL* was indeed up-regulated or expressed ectopically in the mutant line identified in the activation tagging screen.

a

```
>gi|6715465|gb|AF218466.1|AF218466   Activation tagging vector pSKI0715,
complete sequence
          Length = 10450
 Score =  230 bits (116), Expect = 2e-57
 Identities = 124/126 (98%), Gaps = 1/126 (0%)
 Strand = Plus / Plus

Query: 1    tcgccttttcagaaatggataaatagccttgcttcctattatatcttcccaaattaccaa 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct: 8632 tcgccttttcagaaatggataaatagccttgcttcctattatatcttcccaaat-accaa
8690

Query: 61   tacattacactagcatctgratttcataaccaatctcgatacaccaaatcgactctagcg 120
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 8691 tacattacactagcatctgaatttcataaccaatctcgatacaccaaatcgactctagcg
8750

Query: 121  aattcc 126
             ||||||
Sbjct: 8751 aattcc 8756
```

b

```
>gi|6537289|gb|AF187951.1|AF187951   Activation-tagging vector pSKI015,
complete sequence
          Length = 10138
 Score = 1216 bits (613), Expect = 0.0
 Identities = 658/670 (98%), Gaps = 5/670 (0%)
 Strand = Plus / Minus

Query: 186  ttacaattactatttacaattaccatgagcccagaacgacgcccggccgacatccgccgt 245
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9366 ttacaattactatttacaattaccatgagcccagaacgacgcccggccgacatccgccgt
9307

Query: 246  gccaccgaggcggacatgccggcggtctgcaccatcgtcaaccactacatcgagacaagc 305
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9306 gccaccgaggcggacatgccggcggtctgcaccatcgtcaaccactacatcgagacaagc
9247

Query: 306  acggtcaacttccgtaccgagccgcaggaaccgcaggagtggacggacgacctcgtccgt 365
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9246 acggtcaacttccgtaccgagccgcaggaaccgcaggagtggacggacgacctcgtccgt
9187

Query: 366  ctgcgggagcgctatccctggctcgtcgccgaggtggacggcgaggtcgccggcatcgcc 425
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9186 ctgcgggagcgctatccctggctcgtcgccgaggtggacggcgaggtcgccggcatcgcc
9127
```

Figure 3.2. BLAST search results from plasmid A sequence obtained by plasmid rescue from *35Sen::BIL* genomic DNA.
a BLAST search result showing that nucleotide 1 to 126 of the sequence from rescued plasmid A is from the PSKI015 activation tagging vector. **b** The BLAST search from the remainder of the sequence (186 to 425) shows that this represents a different part of the T-DNA, suggesting a tandem insertion of T-DNAs.

a

```
>gi|6537289|gb|AF187951.1|AF187951   Activation-tagging vector pSKI015,
complete sequence
          Length = 10138
 Score =  184 bits (93), Expect = 1e-44
 Identities = 99/102 (97%)
 Strand = Plus / Minus

Query: 1    ataaatngccttgcttcctattanatcttcccaaattaccaatacattacactagcatct 60
            |||||| |||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 9532 ataaatagccttgcttcctattatatcttcccaaattaccaatacattacactagcatct 9473

Query: 61   gaatttcataaccaatctcgntacaccaaatcgactctagcg 102
            |||||||||||||||||||| ||||||||||||||||||||
Sbjct: 9472 gaatttcataaccaatctcgatacaccaaatcgactctagcg 9431
```

b

```
>F17C15  chromo:  5   seqgroup:  ESSA  tigr_asmbl:  67580
              Length = 102897

 Score =  176 bits (89), Expect = 2e-043
 Identities = 115/124 (92%), Gaps = 1/124 (0%)
 Strand = Plus / Minus

Query: 102    gaattcggagcagtgccctaatcttgaaactagcttgnatcattgacatttgnngaagat 161
               |||||||||||||||||||||||||||||||||||| |||||| ||| |||   |||||
Sbjct: 54638  gaattcggagcagtgccctaatcttgaaactagcttgaatcattaacaattgat-aagat 54580

Query: 162    attcatattcattttcatgcaaatattagattcgagngtgacanacataaagtttgtctt 221
               |||||||||||||||||||||||||||||||||  |||||| ||||||||||||||||
Sbjct: 54579  attcatattcattttcatgcaaatattagattcgatagtgacacacataaagtttgtctt 54520

Query: 222    ctta 225
               ||||
Sbjct: 54519  ctta 54516
```

Figure 3.3. BLAST search results from plasmid B sequence obtained by plasmid rescue from *35Sen::BIL* genomic DNA.
a Nucleotide 1 – 120 of the sequence obtained from rescued plasmid B are from the T-DNA. **b** The BLAST search result from the remainder of the sequence (120 – 225) identifies it as *Arabidopsis* DNA contained in BAC F17C15. The junction between T-DNA and *Arabidopsis* sequence corresponds to the *Eco R*I site at position 9431 in F17C15.

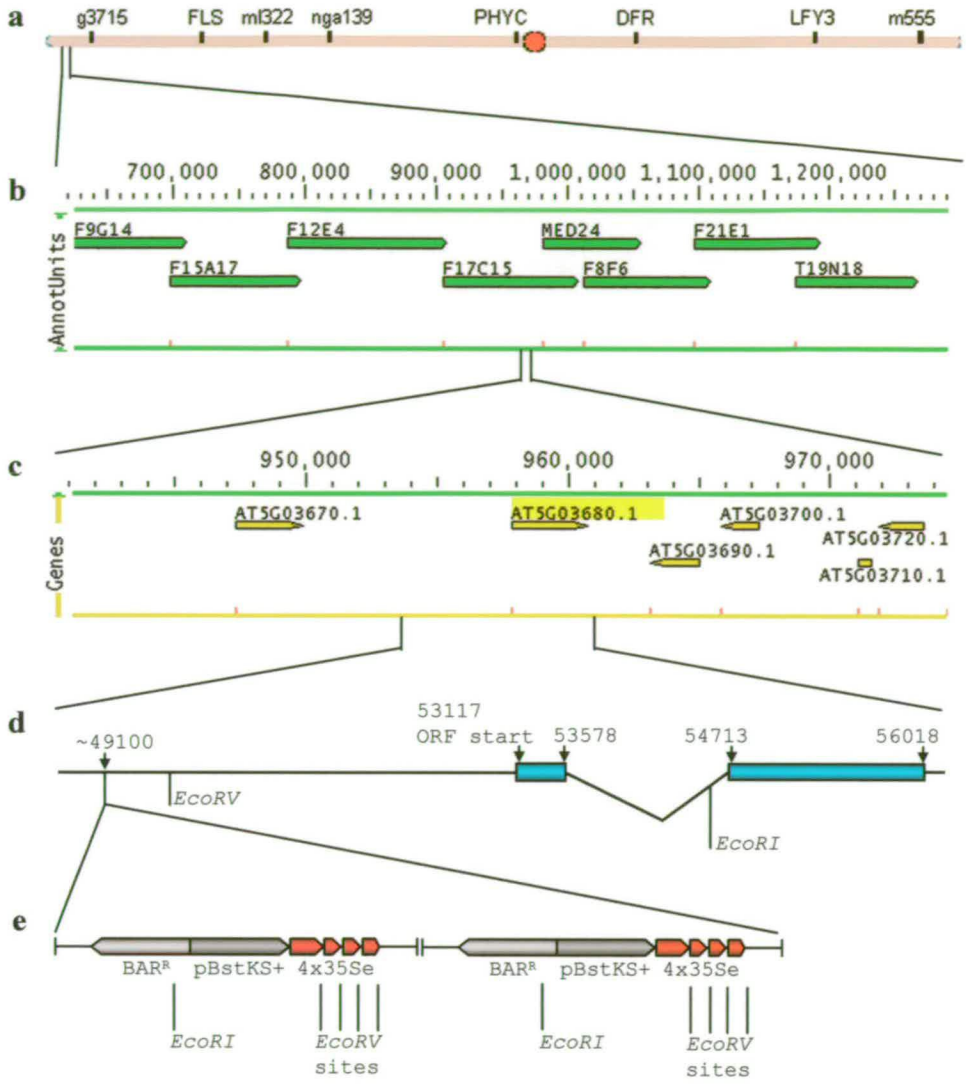


Figure 3.4. Site of activation tagging vector insertion upstream of *PTL* on chromosome 5.

a Chromosome 5. **b** Annotation units (BACs) around the site of the tag. **c** Genes close to vector insert on annotation unit F17C15. *PTL* is highlighted yellow. **d** T-DNA insertion in relation to *PTL.* Green boxes show *PTL* exons, divided by a single intron. T-DNA is approximately 4 Kb upstream of the *PTL* ORF. Numbers represent position on annotation unit. **e.** Adjacent vectors make up the insert. (**a b** and **c** adapted from www.arabidopsis.org/servlets/mapper.)

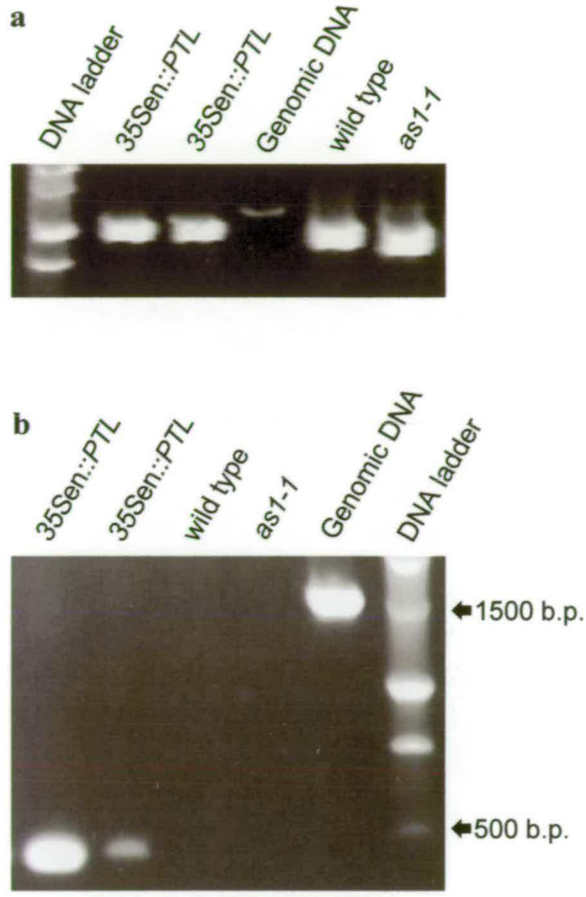


Figure 3.5. RT-PCR showing *PTL* expression in *35Sen::PTL* and wild type leaves.

a *ACTIN2* amplification from cDNA samples used in RT-PCR indicates cDNA samples from different plants are similar. **b** *PTL* expression in two *35Sen::PTL* mutants at different levels presumably due to variable penetrance. *PTL* expression in wild type and *as1-1* leaves is below the level of detection.

3.1.4 *35S$_{en}$::PTL* confers diverse phenotypes when crossed into a Columbia background.

The F2 population generated from the cross between *35S$_{en}$::PTL* and Col-0 (Section 3.2) contained several different phenotypes. About one quarter (8/28) of the plants that survived BASTA, and therefore carried *35S$_{en}$::PTL*, developed *as1* mutant rosette leaves as expected. Of these, three assumed to be homozygous for *35S$_{en}$::PTL* showed adaxial curling at the leaf margins. In the other survivors of BASTA selection that lacked the *as1* mutant phenotype, adaxial curling of the leaf margins was observed in eight plants - i.e. curling was shown by 11 plants in total (39.3%; Fig 3.6). Twelve plants (42.9%) that had neither an *as1* mutant phenotype nor extreme adaxial curling had phenotypes similar to the F1 plants - i.e. serrated margins and slight adaxial curling. As no obvious Mendelian ratio was observed for the F2 leaf phenotypes, the *35S$_{en}$::PTL* mutation was considered to have variable penetrance. Furthermore, since leaves in *as1-1 35S$_{en}$::PTL* mutants had similar defects at the margins to *35S$_{en}$::PTL* mutants, the *as1* and *35S$_{en}$::PTL* leaf phenotypes were likely to be additive, suggesting that *AS1* and *PTL* do not normally interact in leaf development. Because *ptl* mutations do not affect leaves, *PTL* has not previously been assigned a role in leaf development. However, since the activation tagging T-DNA is thought to confer up-regulation mainly from active promoters, a redundant role for *PTL* in leaf development appears a possibility.

The F2 population also segregated a distinct phenotype in the primary inflorescence. The distance between nodes was greatly reduced in 13 plants (46.4 %) leading to extremely short stature (Fig 3.7). Nine of the 13 plants also had the strong curled leaf phenotype assumed to result from homozygosity of *35S*$_{en}$::*PTL*, but the remaining four had leaves with the *as1-1* phenotype. One explanation for this is that a single copy of *35S*$_{en}$*PTL* in an *as1* mutant background can cause the same inflorescence phenotype as two copies of *35S*$_{en}$*PTL* in an *AS1*$^{+}$ background.

Six of the surviving F2 population (21.4%) generated flowers with larger petals or increased petal number. Closer investigation of flowers with these phenotypes also revealed an increase in carpel number (Fig 3.8). These features were observed in plants with both weak and strong leaf or inflorescence phenotypes. This suggested that the floral phenotype was not dependent on the dose of *35S*$_{en}$::*PTL*, as proposed for leaf and inflorescence development. Plants were also observed with *as1* mutant petals despite the presence of *35S*$_{en}$::*PTL*, in stark contrast to the plant identified initially in the mutagenesis screen which had a suppressed petal phenotype. This suggested that *AS1* and *PTL* do not interact, an observation corroborated by experiments in which loss-of-function *ptl* mutations in an *as1-1* mutant background gave an additive phenotype (Philip Brewer, personal communication). The *35S*$_{en}$::*PTL* mutation was originally isolated with a view to uncovering a possible target of *AS1*. The lack of interaction suggested that *PTL* was unlikely to be a target of *AS1*..

Non-Mendelain ratios of phenotypes were a feature of populations segregating *35S*$_{en}$*::PTL*. One explanation is that the T-DNA insertion might be silenced (e.g. by PTGS). An alternative is involvement of a dominant modifier of *ptl*, *petal loss modifier* (*pmd-1d*), that has been identified in L*er* (the predominant background of *as1-1*). The *pmd-1d* allele causes a marked increase in the number of petals generated by *ptl* mutant plants (up to eight petals per flower early in development (Griffith *et al.*, 1999).

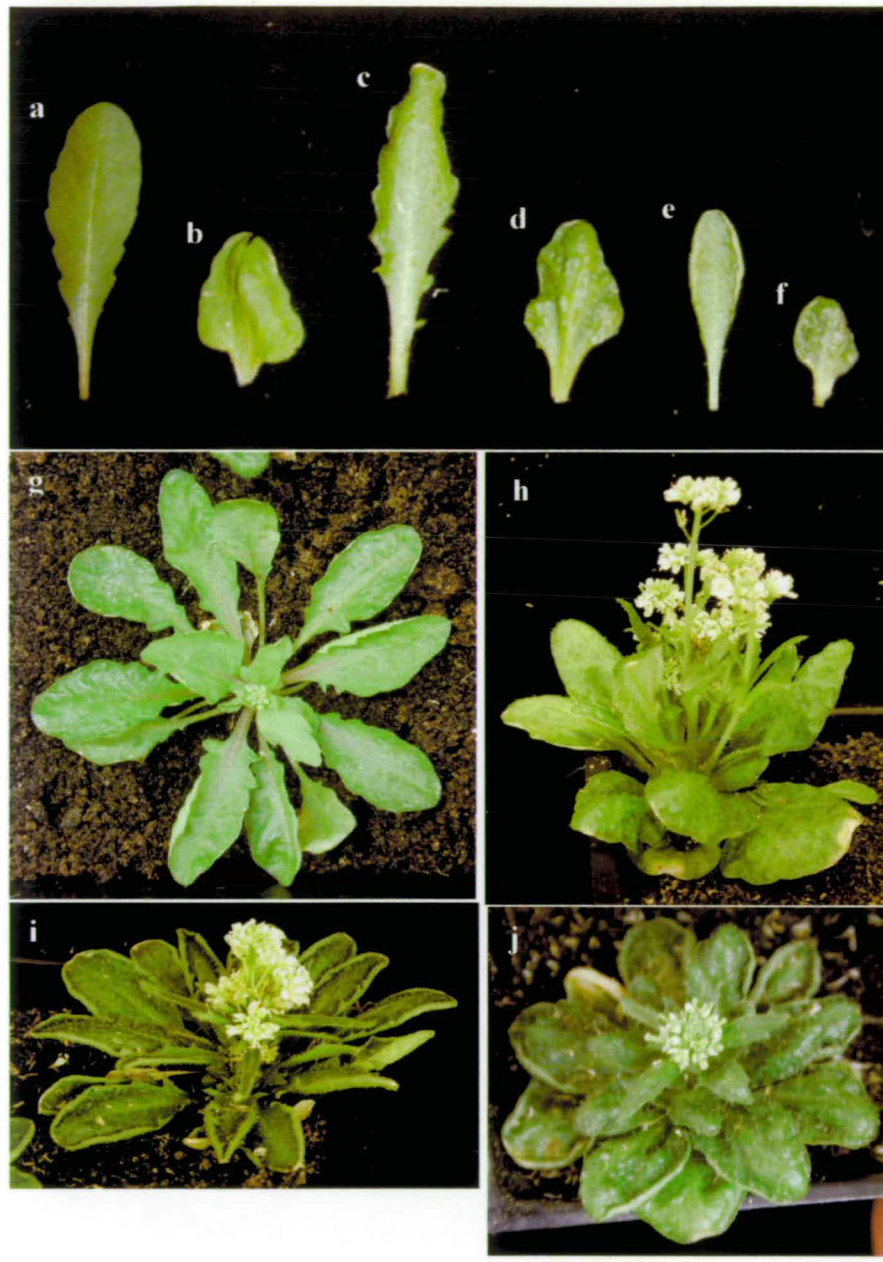


Figure 3.6. Rosette leaf phenotype of *35Sen::PTL* in *as1* mutant and wild type background.
a wild type. **b** *as1-1*. **c and g** *35Sen::PTL/+ AS1*$^{+}$. Leaves develop serrations and curl adaxially at the margins. **d and h.** *as1-1 35Sen::PTL/+*. Leaves are similar to *as1-1*. **e and i** *35Sen::PTL*. Severe curling occurs at the margins and leaves are not elaborated to normal size. **f and j** *as1-1 35Sen::PTL*. Leaves are smaller than normal *as1* mutants. Margins curl adaxially.

Figure 3.7. Variation in height and internode length of *35Sen::PTL* plants.
a wild type. **b** *35Sen::PTL/+* mutant of similar height to wild type but with highly variable internode length (arrows). **c** *35Sen::PTL* mutant with reduced apical dominance. **d** *35Sen::PTL* mutant with variable internode length (arrows), and significantly taller than wild type.

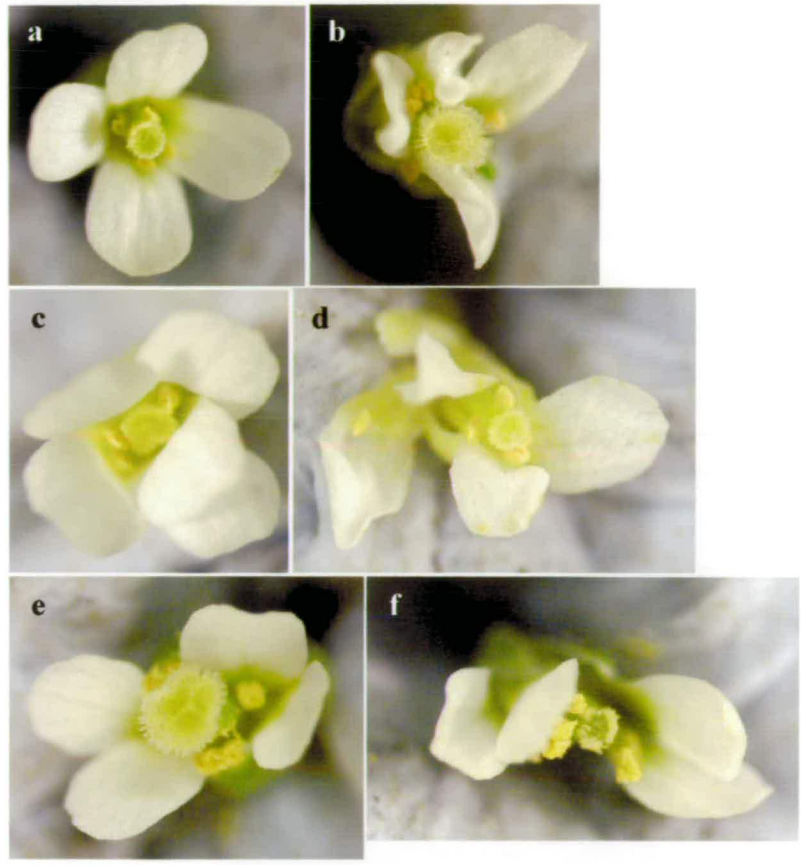


Figure 3.8. Floral phenotypes of *35Sen::PTL* mutants crossed into the Col-0 background.
a Wild type flower. **b** *as1-1* mutant flower. **c** *35Sen::PTL* mutant with 5 petals. **d** *as1-1 35Sen::PTL* mutant with unsuppressed floral phenotype. **e** *35Sen::PTL* mutant with increased carpel number. **f** *35Sen::PTL* mutant with normal petal and carpel number.

3.2 DISCUSSION.

3.2.5 *PTL* encodes a GT transcription factor required for normal petal development.

PTL encodes a member of the GT transcription factor family. The founding member of the family, GT-1, was identified as a protein that bound to light-regulated promoters containing a sequence that could confer positive light regulation on otherwise light-insensitive promoters. *GT-1*, however, is not itself light-responsive (Lam and Chua, 1990). The GT-2 subfamily, to which PTL belongs, is closely related to, but distinct from, the GT-1 family. Its members have two MYB domains and function as transcriptional activators (Ni *et al.*, 1996).

In wild-type plants, *PTL* is expressed between developing sepals prior to petal initiation and is thought to inhibit growth between sepal primordia, providing a limited domain for petals to arise (Brewer *et al.*, 2002). *PTL* is also likely to provide information required for petal orientation as about 40% petals that are developed in *ptl* mutants have their adaxial surface oriented in a different direction. This phenotype is enhanced by the B function homeotic mutations *pistillata* (*pi*) and *apetala3* (*ap3*) as in *ptl-1 ap3-3* double mutants and *ptl-1 pi-1* double mutants second whorl organs are almost all completely reversed in orientation (Griffith *et al.*, 1999). The most striking characteristic of *ptl* mutants is however the petal number. Mutants develop either two or three petals in early flowers, but the mean petal number falls such that most late flowers have none. This suggests that *PTL* is required for petal initiation and therefore that it might be excluded from the floral meristem in much same

way as *AS1* is excluded from the SAM. Interestingly, ~25% of *ptl* mutant petals are either trumpet-shaped or tubular, suggesting a role for *PTL* in petal polarity as well as orientation. A similar phenotype is seen in leaves of Antirrhinum *phan* mutants which lack activity of the *AS1* orthologue (Waites and Hudson, 1995), and very rarely in *as1* mutant leaves.

Defects in *ptl* mutants also occur in the first and third floral whorls, as well as the second whorl, although the phenotypes are much less severe. In the first whorl, sepals are occasionally fused and one in five develops carpelloid structures on their edges. Third whorl defects are extremely infrequent and manifest as an additional stamen, or a stamen with a single filament, but two anthers.

A role for *PTL* in specification of petal development is consistent with the phenotypes observed in the *35Sen::PTL* mutants. An increase in the domain of *PTL* expression is likely to cause an increase in both petal size and petal number (essentially the converse of the *ptl* loss-of-function phenotype) and this is consistent with the reported domain of *PTL* expression between sepal primordia. The suggestion that *PTL* encodes a growth repressor (Brewer *et al.*, 2002) is harder to understand. In this case an increase in *PTL* expression should increase growth repression between sepals, including the floral meristem and should result in smaller or fewer petals. The increase in petal number and size argues against this role, but supports a role in setting a domain for petal development.

The *35S*$_{en}$*::PTL* phenotype has uncovered a wider range of expression than previously described for *PTL*. When *PTL* expression is up-regulated by the *35S* enhancer, carpel number in the fourth whorl is increased, suggesting that *PTL* normally functions here. The leaf and inflorescence phenotype of *35Sen::PTL* plants also suggest that *PTL* is active outside the flower and earlier in development. These roles might not be apparent from loss-of-function phenotypes because of redundancy of *PTL* due to overlapping expression of related genes in these tissues.

3.2.6 The role of *PTL* in *Arabidopsis* development remains obscure

With the exception of the B function floral homeotic mutants and the modifier *pmd*, *ptl* has not been found to interact with other developmental mutations. The *35Sen::PTL* phenotype suggests that *PTL* is involved in vegetative development, but mechanisms underlying this function are difficult to determine because loss-of-function mutants lack a vegetative phenotype and gain-of-function mutants have a broad range of phenotypes. Disrupted orientation of second whorl floral organs in *ptl* loss-of-function mutants might, however, give clues about what is happening in the leaves of *35Sen::PTL*. Loss of polarity or orientation of *ptl* mutant petals suggests that they have defects along their adaxial/abaxial axis. One explanation for this is that *ptl* mutant petals get incorrect abaxial or incorrect adaxial signals or are unable to interpret them correctly. The upward curling of the lamina observed

in *35Sen::PTL* is also a feature of other mutants in which leaf polarity is affected. For example, loss of *KANADI1* gene function causes adaxial curling of the lamina due to an increase in the number of abaxial cells as these take on adaxial features. *KANADI1* is expressed abaxially, and is thought to confer abaxial identity (Kerstetter *et al.*, 2001).

More severe disruptions to organ polarity result in loss of organ growth. For example, radiallised floral organs are caused by loss of the *FILAMENTOUS FLOWER* (*FIL*) gene (Chen *et al.*, 1999; Sawa *et al.*, 1999), a member of the *YABBY* family which is expressed abaxially in lateral organs (Siegfried *et al.*, 1999). Although similar defects are seen in *ptl* mutants, they are not a feature of *35Sen::PTL* flowers. Therefore the *35Sen::PTL* phenotype is unable to reveal more of the potential role of *PTL* in organ polarity.

4.0 LOSS-OF-FUNCTION MUTAGENESIS IN AN *as1* MUTANT BACKGROUND

4.1 RESULTS.

4.1.1 Loss-of-function mutagenesis in an *as1* mutant background.

If *knox* mis-expression was solely responsible for the *as1* mutant phenotype, a reduction of *knox* expression in *as1* mutants should result in a suppressed *as1* phenotype. However, in *as1-1 bp* double mutants, the *as1* leaf phenotype remains strong. Rosettes are tightly arranged and leaves have asymmetric lobes despite the absence of functional *BP* and therefore its expression in the lamina. This is also the case with the other available *knox* mutations. *as1 knat2* double mutants, *as1 stm* double mutants and *as1 bp knat2* triple mutants also maintain the *as1* mutant rosette phenotype. The available evidence therefore suggests that there are other targets of *AS1* that are responsible for the *as1* mutant phenotype, with the one caveat that no *knat6* mutant is available that has allowed testing of an *as1 bp knat2 knat6* quadruple mutant. It therefore remains a possibility that *as1* mutant leaves would be suppressed in this background. In order to determine what other factors might be involved in the phenotype of *as1* mutants, a modifier screen was carried out in an *as1* mutant background. Approximately 17,000 *as1-1* seeds were subjected to EMS mutagenesis and M1 plants allowed to set seeds. M2 seeds were harvested from pools of ~five M1 plants and screened for plants that had a more or a less severe *as1* phenotype. EMS causes point mutations that usually result in loss of function, so the objective of the screen was to identify genes acting redundantly with *AS1* as recessive or semi-dominant enhancers of *as1*, or identification of genes acting in parallel or downstream of *BP* and the other *knox* mutants as suppressers of *as1*.

4.1.2 *symmetrica* is a dominant suppresser of *as1-1*

The *symmetrica* (*sym*) mutant was identified in the M2 population as a suppresser of *as1-1* (Fig. 4.1). Rosette leaves of *as1-1* mutants are reduced, have thick petioles, basal lobes and a rough appearance (Fig. 3.1). In contrast, *sym* mutants had rosette leaves of similar size to wild-type. Suppression of the *as1-1* lateral organ phenotype extended to cauline leaves and flowers. Rarely *sym* mutant plants had a small asymmetric lobe at the proximal end of lamina.

To confirm that *sym* suppressed the *as1* phenotype, the *as1* gene was amplified by PCR from *sym* plants with a wild-type phenotype. Direct sequencing of the PCR product detected a single sequence with the nucleotide deletion characteristic of *as1-1*, confirming that plants were *as1-1* homozygotes and therefore that the *as1* phenotype was suppressed.

To obtain *sym*/+ heterozygotes, seeds from potential M2 siblings of the *as1 sym* mutant that had an *as1* mutant phenotype were sown. One M3 family consisted of *as1* and wild-type phenotypes in a ~3:1 ratio (38 *as1* mutants: 11 wild-type) consistent with suppression being caused by a single recessive mutation, *sym*, that was heterozygous in the parent.

To further test whether *sym* was recessive and therefore likely to be a loss-of-function mutation, *as1-1 sym* double mutants were crossed to *as1-1a*, which carries the *as1-1* mutation in its original Col-0 background. The F1 plants had

an intermediate phenotype and the F2 population consisted of two discernable classes (1) *as1* mutants and (2) plants of wild-type or intermediate phenotype, which were considered as one class - suppressed *as1* - since phenotypic differences could not reliably separate plants along the lines of expected genotype (26 classical *as1* mutants, 74 of suppressed *as1* phenotype). In plants with the intermediate phenotype, laminae were asymmetric but no longer heart shaped (Fig. 4.1). Leaf margins were curled abaxially as for *as1* mutants. Petioles were reduced in length relative to wild-type, but to a lesser extent than in *as1-1*. Petioles were wider than wild-type, as in *as1* single mutants. This suggested that the *sym* mutation was semi-dominant, at least in this background, and raised the possibility that complete suppression involved mutations in more than one gene.

The *as1 sym* double mutant was also back-crossed to its *as1-1* parent with mixed Col-L*er* background. F1 plants had the intermediate phenotype, consistent with the presence of more than one mutation. There was insufficient time to generate an F2 population from this cross to test the number of mutations further. However, mapping of the *SYM* locus provided evidence for a second modifier mutation, unlinked to *SYM*.

Figure 4.1. *symmetrica* suppresses the *as1-1* mutant phenotype in a semi dominant manner. **a** Wild type rosette. **b** *as1-1*. *as1-1* rosette leaves are small, heart-shaped with small lobes. Absent petiole **c** *as1-1 sym* double mutant. Unlike *as1-1* single mutants, the double mutant forms petioles and lamina of normal size. The shape of the lamina is altered in some plants with a small proximal lobe. **d** Plant carrying an *as1-1/as1-1 sym/SYM* genotype. features are intermediate with respect to *as1-1* and *as1-1 sym.* leaves are neither heart-shaped, nor symmetric. Petioles are present. **e** *as1-1/as1-1a sym/SYM.* When crossed into a columbia background, *sym* is a stronger suppresser of *as1*.

4.1.3 *SYMMETRICA* is linked to *AS1* and has no single mutant phenotype

To determine the phenotype of *sym* in a wild-type, *AS1*$^+$, background, Col-0 wild-type plants were crossed to *as1-1 sym* homozygotes. F1 plants were wild-type in appearance. If *sym* behaved as a semi-dominant suppresser of *as1*, the expectation was that $^1/_{16}{}^{th}$ (6.25%) of the F2 progeny would be homozygous for *sym* and *as1* and therefore fully suppressed while $^1/_8{}^{th}$ (12.5%) would be homozygous for *as1* and heterozygous *sym*/+ and therefore have the intermediate phenotype. However, only 11 single *as1* mutants were found in 730 plants (1.51%). The simplest explanation for this result was that the *AS1* and *SYM* loci were linked with a map distance between the two of around 3 cM.

The population, and larger F2 populations from the same cross, yielded no plants of novel phenotype, suggesting that *sym* homozygotes (which are expected to occur at a similar frequency to *as1 SYM*$^+$ mutants), have no visible mutant phenotype, suggesting a redundant role for *SYM*. The possibility also remained that the homozygous *sym* mutation is lethal in an *AS1*$^+$ background.

4.1.4 *sym* is located south of *AS1* on Chromosome 2

The *as1-1 sym* cross to Col-0 cross suggested that the *SYM* locus was closely linked to *AS1*. To confirm that the *as1-1 sym* phenotype was caused by a

mutation at a single locus linked to *AS1*, *as1-1 sym* homozygotes were crossed to *as1* mutants in different ecotypic backgrounds. F2 populations were generated from *as1-1 sym* x *as1-mag* (En background) or *as1-1 sym* x *as1-1a* (Col-0 background). As before, these segregated in a ~1:2:1 ratio, wild-type like, intermediate and severe *as1* phenotypes, assumed to be *sym/sym*, SYM^+/sym and SYM^+/SYM^+ homozygotes, respectively. Because the intermediate phenotype could not be distinguished from the completely suppressed phenotype with certainty, 40 putative SYM^+/SYM^+ F2 homozygotes from the *as1-mag* cross and 46 from the *as1-1* cross were analysed with SSLP markers covering the whole genome. For unlinked markers SSLP genotypes were expected to occur in a 1:2:1 ratio of Col-0 or En homozygotes, heterozygotes and L*er* homozygotes (Fig. 4.2). The observed ratios were tested for deviation from this ratio that suggested linkage, using the chi squared test (Sokal and Rohlf, 1995). Polymorphic markers on the upper arm of Chromosome 2 and both arms of Chromosomes 1, 3, 4 and 5 showed no significant difference from segregation ratios expected of loci unlinked to *SYM* (Table 4.1; Fig. 4.4). Only *nga1126* and *nga361*, north of *AS1* on the bottom arm of Chromosome 2 deviated in their segregation ratios in the *as1-1 sym* x *as1-1a* population (Table 4.1; Figs. 4.2 and 4.3). This suggested that the *sym* mutant phenotype was indeed associated with a single locus linked to *AS1*. *nga361* demonstrated tighter linkage to *SYM* than *nga1126* (14 and 18 recombinants, respectively).

A further segregation pattern was observed in a different map position that significantly deviated from the 1:2:1 segregation pattern. This occurred at marker *nga158*, 26 cM from the top of chromosome 5 (Fig. 4.4) for which more L*er* alleles were detected in the strong *as1* mutants compared to En alleles (61 compared to 11, respectively).

None of the SSLP polymorphisms reported for Col-0 and L*er* on the lower arm of Chromosome 2 proved to be polymorphic between *as1-1* and *as1-mag*. Furthermore, markers in the region immediately south of *nga361* in plants generated from *as1-1 sym* (mixed L*er*/Col-0) and *as1-1a* (Col-0) parents were monomorphic suggesting that this part of the Chromosome in the *as1-1* background had come from the Col-0 ecotype when the *as1-1* mutation was introgressed into L*er*. A third cross was therefore carried out to map the region south of *nga361*. *as1-12* (L*er*) was crossed to *as1-1 sym* (mixed L*er*/Col) and 94 F2 plants with a severe *as1* phenotype were analysed for segregation of polymorphic SSLP markers. In this population, *SYM* was found to be linked to markers *CER449030*, less than 1 cM south of *AS1*, and *nga168*, approximately 4 cM south of *AS1* (Table 4.1; Fig. 4.3). More recombination events (17) had occurred between *CER449030* and *SYM* than had occurred between *nga168* and *SYM* (13), suggesting that *nga168* was the closer of the two markers to *SYM*. All recombinant chromosomes at *nga168* were also recombinant at *CER449030* indicating that *SYM* lay to the south of both markers.

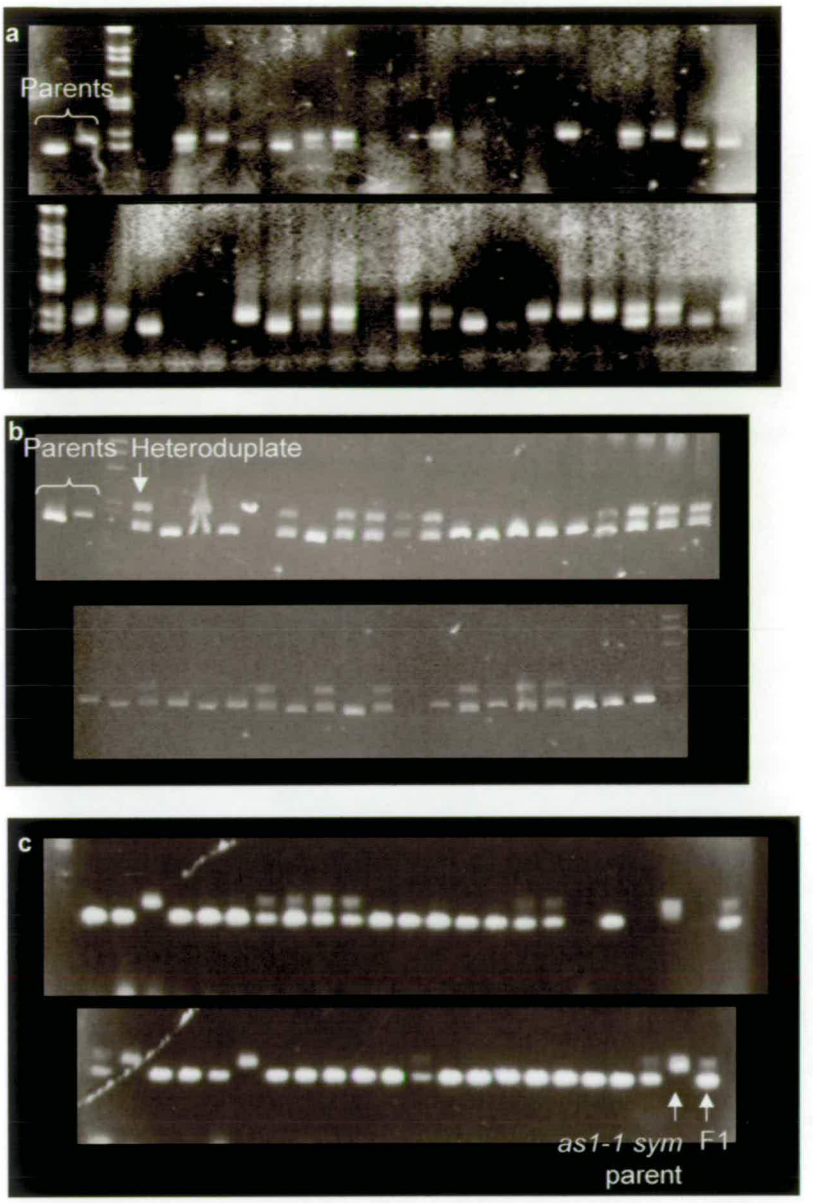


Figure 4.2 Gel photographs showing 3 types of segregation pattern of makers in *SYM*/+ plants from mapping crosses.
a Normal segregation of marker at nga248 (Ch 1), unlinked to *SYM*. 25% of plants are homozygous for each allele which is represented by a single band (8 plants with *as1-mag* (En) and 9 *as1-1 sym* (L*er*/Col), respectively). 50 % of plants are heterozygotes (12 plants). **b** Segregation of heteroduplates at MBK5 (Ch 5), unlinked to *SYM*. Homozygous alleles are not scoreable, but heterozygotes form secondary structures that result in visible differences in band migration allowing camparison of homo- to heterozygotes which are each expected at 50 %. **c.** Segregation of markers at nga361 (Ch 2), linked north of *AS1*. Markers do not fall into the normal 1:2:1 ratio indicating that the marker is close to *SYM* and recombination between *SYM* and the marker is less likely due to the close proximity of the two.

Marker	Chromo-some	Enkheim (*as1-mag*)	L*er*/Col (*as1-1*)	Hetero-zygote	χ^2 Value
nga248	1	8	9	12	0.92
nga128	1	10	10	14	1.12
nga692	1	12	6	18	2.0
nga1145	2	8	11	18	0.98
ATHCHIB	3	8	5	17	1.12
nga12	4	8	5	10	0.57
nga1139	4	12	8	18	0.95
nga139	5	7	11	19	0.89
nga129	5	8	5	15	1.67
MBK-5	5	19 (monomorphic)		18 (as hetero duplet)	0.03
Marker	**Chromo-some**	**Columbia (*as1-1a*)**	**L*er*/Col (*as1-1*)**	**Hetero-zygote**	**χ^2 Value**
nga361	2	26	4	10	34.2
nga1126	2	24	7	11	28.47
fus 6.2	3	11	9	19	0.31
ciw4	3	10	9	21	0.15
Marker	**Chromo-some**	**L*er* (*as1-12*)**	**L*er*/Col (*as1-1*)**	**Hetero-zygote**	**χ^2 Value**
CER449030	2	67	0	17	136.60
nga168	2	63	0	13	113.35

Table 4.1 Segregation of unlinked markers in *SYM* plants from *as1-1 sym* x *as1-mag* and *as1-1 sym* x *as1-1a* F2 populations used to map *SYM* to chromosome arm 2.

Segregation pattern of bands associated with each ecotype segregating in the *as1* mutants of the F2 population. No linkage to *SYM* was uncovered using polymorphic markers between *as1-1 sym* and *as1-mag* as polymorphisms were not found between these backgrounds on all the chromosome arms. Linkage between polymorphic molecular markers and *SYM* were discovered in the *as1-1 sym* x *as1-1a* and *as1-1 sym* x *as1-12* (highlighted yellow).

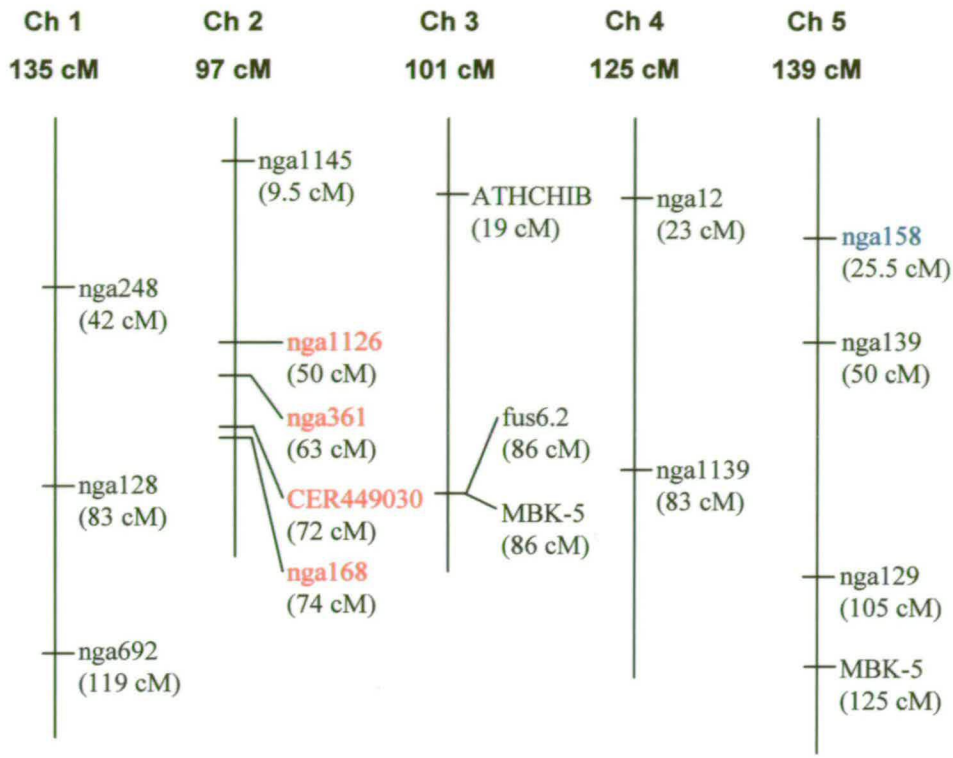


Figure 4.3 Positions of molecular markers used for mapping *SYM*

Map of *Arabidopsis* chromosomes with positions of markers used to map *SYMMETRICA* to the bottom of chromosome 2. Markers linked to *SYM* are marked red. Anomalous segregation, where *nga158* demonstrated linkage with a feature on the En chromosome associated with *as1* mutants in the *as1-1 sym* x *as1-mag* F2 is marked blue.

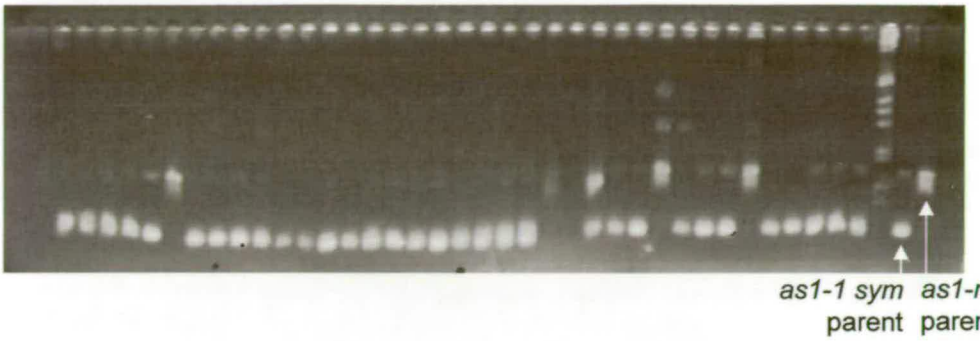


Figure 4.4 Marker nga158 at the top of chromosome 5 demonstrating linkage to a feature that predominantly segregates with the *as1* mutation.

The gel shows a segregation pattern that significantly deviates from the 1:2:1 expected. Only 1 heterozygotes and three En homozygotes are observed.

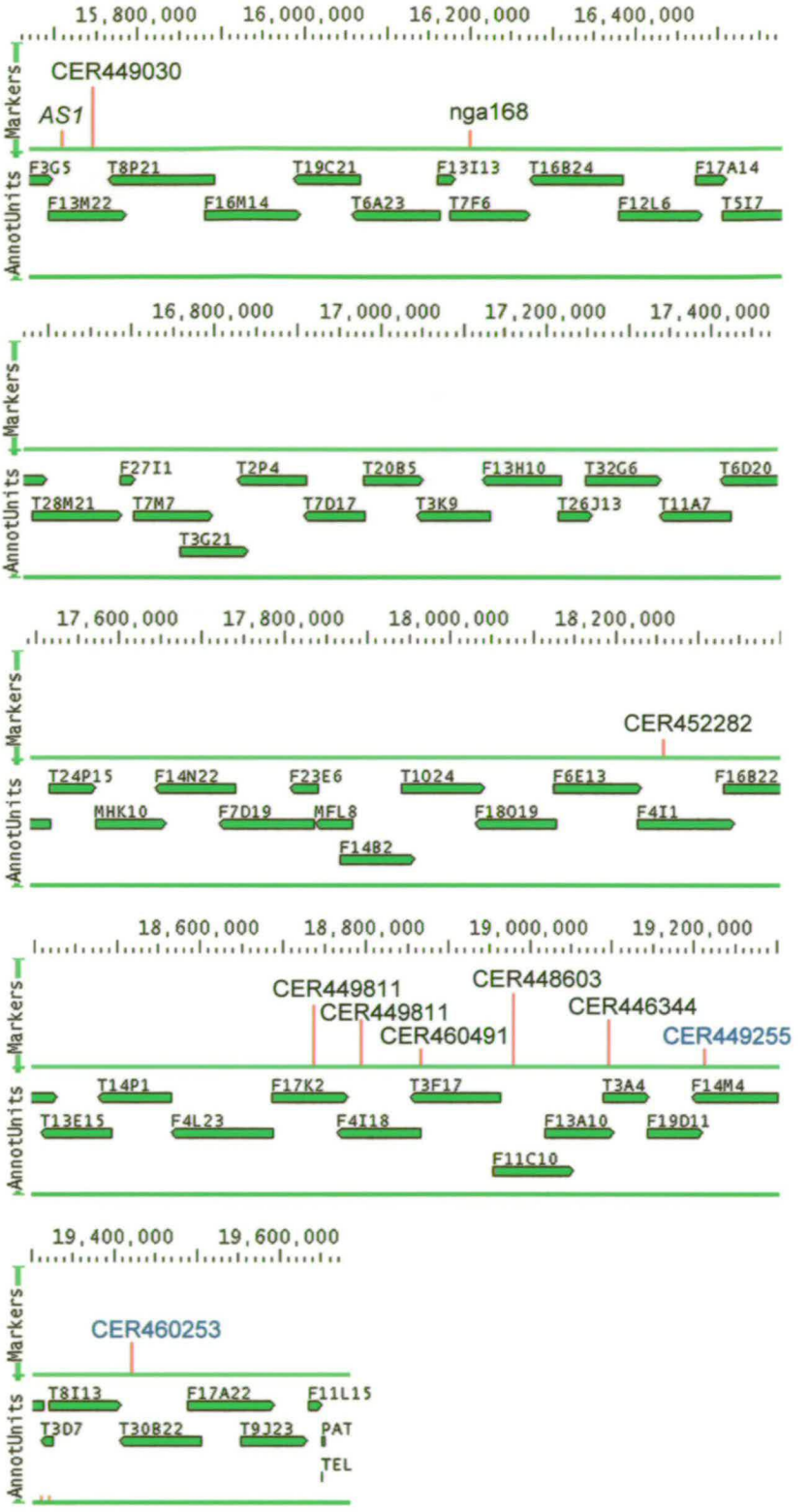


Figure 4.5 Positions of markers used for fine mapping *SYM*

Arabidopsis chromosome 2 from ~72 cM to the telomere with positions of markers used to map *SYMMETRICA*. Markers south of *SYM* are in blue.

However, markers south of *nga168* did not prove to be polymorphic in this background suggesting that the breakpoint of the introgression occurred between them and *nga168* and therefore that the region south of *nga168* in the *as1-1* background originated from L*er*. A more accurate map position for *SYM* was therefore obtained using the *as1-1 sym* (mixed L*er*/Col) x *as1-1a* (Col) F2 population.

4.1.5 An accurate map position for *SYMMETRICA*

In order to reduce the number of genes likely to be encoded by *SYM*, 44 *as1* mutants from the *as1-1 sym* (mixed L*er*/Col) x *as1-1a* (Col) F2 population were analysed at positions south of *nga168* for recombination events between molecular markers of known position and *SYM* (Fig. 4.5). In this set of experiments, the marker *CER452282*, located on annotation unit F4I1 approximately 2000 Kb south of *nga168* was tested first. Of the 44 plants tested, seven had recombinations between *CER452282* and *SYM*. These plants were used test for recombination at markers *CER449811*, *CER452340*, *CER452377*, *CER448603* and *CER449255* on annotation units F17K2, F4I18, T3F17, F11C10 and F14M4, respectively at approximately 550 Kb, 600 Kb, 650 Kb, 750 Kb and 1000 Kb south of *CER4542282* (Fig. 4.5). Of the seven recombinants at *CER4542282*, 4, 3, 2, 2, and 0 were also recombinant for *CER449811*, *CER452340*, *CER452377*, *CER448603* and *CER449255*, respectively. This suggested that *SYM* lay south of F11C10. Because no recombinations were present between the marker on F14M4 and *SYM*, a

further 282 *as1* mutants from the F2 population were screened for heterozygosity of the F14M4 marker. Two of these were found with a recombination event between F14M4 and *SYM*. In order to determine whether *CER449255* (on F14M4) was north or south of *SYM*, two more markers were tested. One marker, *CER446344*, lay between F11C10 and F14M4 on annotation unit T3A4 and the other, *CER460253* was located south of F14M4 on annotation unit T30B22. The two recombinants at *CER448603* (on F11C10) and the two at *CER449255* (on F14M4) were tested for presence or absence of recombination events between the new markers and *SYM*. At *CER446344* on T3A4, two plants had recombination events between the marker and *SYM*. These recombination events were also observed in *CER448603* (on F11C10) but not at *CER449255* (on F14M4), suggesting *SYM* lies at a location south of *CER446344*. Conversely, the two plants with recombinations between *CER449255* (on F14M4) and *SYM* were also recombinant between *CER460253* on T30B22, suggesting that *SYM* lies to the north of *CER449255* (on F14M4). The gap of approximately 120 Kb between the markers contains 35 predicted ORFs, one of which is likely to encode *SYMMETRICA*.

One factor, which may have caused contamination of the mapping population and therefore alter the map position is the modifier of *sym* identified in the original M2 population which made *sym* recessive, rather than dominant as it appears in other backgrounds. If this modifier was present in a proportion of the classical *as1* mutants chosen for mapping, contamination of the mapping

population with *sym* heterozygotes is a consideration. However the number of *as1* mutants in the mapping populations does not suggest that this was the case.

4.1.6 Candidate *SYM* genes

Of the 35 genes, 12 encode proteins of known function that were considered unlikely to be *SYM*. These included genes involved in regulation of the circadian clock, cytochrome P450, and a putative zinc transporter. Twelve genes are described as encoding hypothetical, unknown or expressed proteins (www.arabidopsis.org) and while these genes cannot be discounted, a number of good candidates were present in the genes of known function. The best candidates included an auxin regulated protein, two protein kinases, a putative RNA binding protein, a ubiquitin extension protein and several transcription factors. One candidate (At2g46770) stood out from the rest because it encoded a NAM (NO APICAL MERISTEM)-like protein that was closely related to *CUC2*. This gene was a particularly attractive candidate as *CUC* genes have been shown to promote *knox* gene expression (Hibara *et al.*, 2002). A further test was undertaken to determine whether the candidate gene was likely to be *SYM*. RT-PCR was carried out on wild-type, *as1-1* mutants and *as1-1 sym* double mutants in order to test for changes in At2g46770 expression. The gene was found to be expressed in meristem-enriched tissue in wild-type plants and in *as1-1* mutants, but not in *as1-1 sym* double mutants (Fig. 4.6), confirming At2g46770 as a strong candidate.

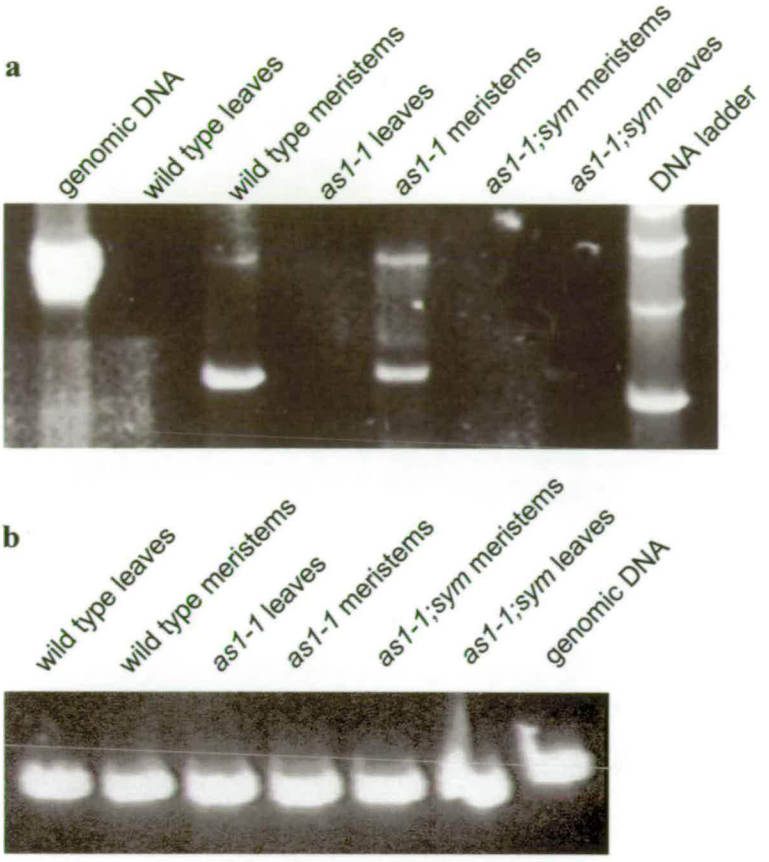


Figure 4.6 RT-PCR showing that At2g46770 expression is not detected in *as1-1 sym* double mutants.

a RT-PCR indicates that At2g46770 is expressed in both wild type and *as1* mutant meristems, but not in *as1-1 sym* double mutants. **b** RT-PCR using *ACTIN2* primers indicates that cDNA preparations are equivalent.

4.1.7 *SYMMETRICA* is required for *KNOX* misexpression in *as1* mutants

The *knotted1*-like homeobox (*KNOX*) genes *BP, KNAT2 and KNAT6* are mis-expressed in *as1* mutant leaves (Fig.. 4.7). To test whether *sym* could also suppress ectopic *KNOX* expression in addition to modifying the *as1* phenotype, *KNOX* expression patterns were investigated in suppressed *sym as1* mutants. RNA was extracted from leaves and apices of *as1-1 sym* double mutants, *as1* single mutants and wild-type and used in cDNA synthesis. RT-PCR was carried out using three different primer pairs for amplification of *BP*, *KNAT2* or *KNAT6* transcripts using equal quantities of cDNA template confirmed by amplification of the ubiquitously expressed *ACTIN2* gene (Fig. 4.7). All amplified cDNA regions spanned introns and genomic DNA was included as a control for amplification of genomic DNA. As expected, the *KNOX* genes were expressed in all apical samples and in *as1-1* leaves. Expression was low or absent from wild-type leaves. In *as1-1 sym* leaves, the *KNOX* genes were expressed ectopically to a significantly lower degree than in *as1* single mutants, despite the absence of AS1 activity (Fig. 3.5). No ectopic expression of *STM* was detected in any genotype. *SYMMETRICA* therefore appeared to be needed for ectopic *KNOX* gene expression in *as1* leaves.

4.1.8 Reporter gene analysis of *knox* misexpression in *as1-1 sym* double mutants

To test whether *SYM* promoted *KNOX* gene transcription or post-transcriptional stability of *KNOX* transcripts constructs consisting of the promoters of *BP* and *KNAT2* driving expression of the *UidA* (GUS) gene were introduced into *as1* and *as1 sym* backgrounds by crossing. In *as1* mutant leaves and cotyledons, GUS expression from the *BP* promoter occurred mainly in the vasculature along the full length of the midrib, in the hydathodes, and in sinuses between lobes where present (Fig. 4.8), as reported previously (Ori *et al.*, 2000). In wild-type plants however, expression was completely absent from leaves and cotyledons. To determine how the *sym* mutation effects *BP* expression in *as1* leaves, pollen from p*BP::GUS* lines in an *as1* mutant background were used to pollinate *as1 sym* stigmata. F1 plants were screened for presence of the transgene and F2 plants were analysed for GUS staining. At the hydathodes, the p*BP::GUS* expression was similar to that in *as1* single mutants, while in the midvein a large reduction of ectopic expression was observed. In contrast to *as1* plants, where staining occurs along the entire proximal-distal axis, *as1 sym* double mutants stained for GUS only in petioles and staining did not extend as far as the leaf lamina in either cotyledons or leaves.

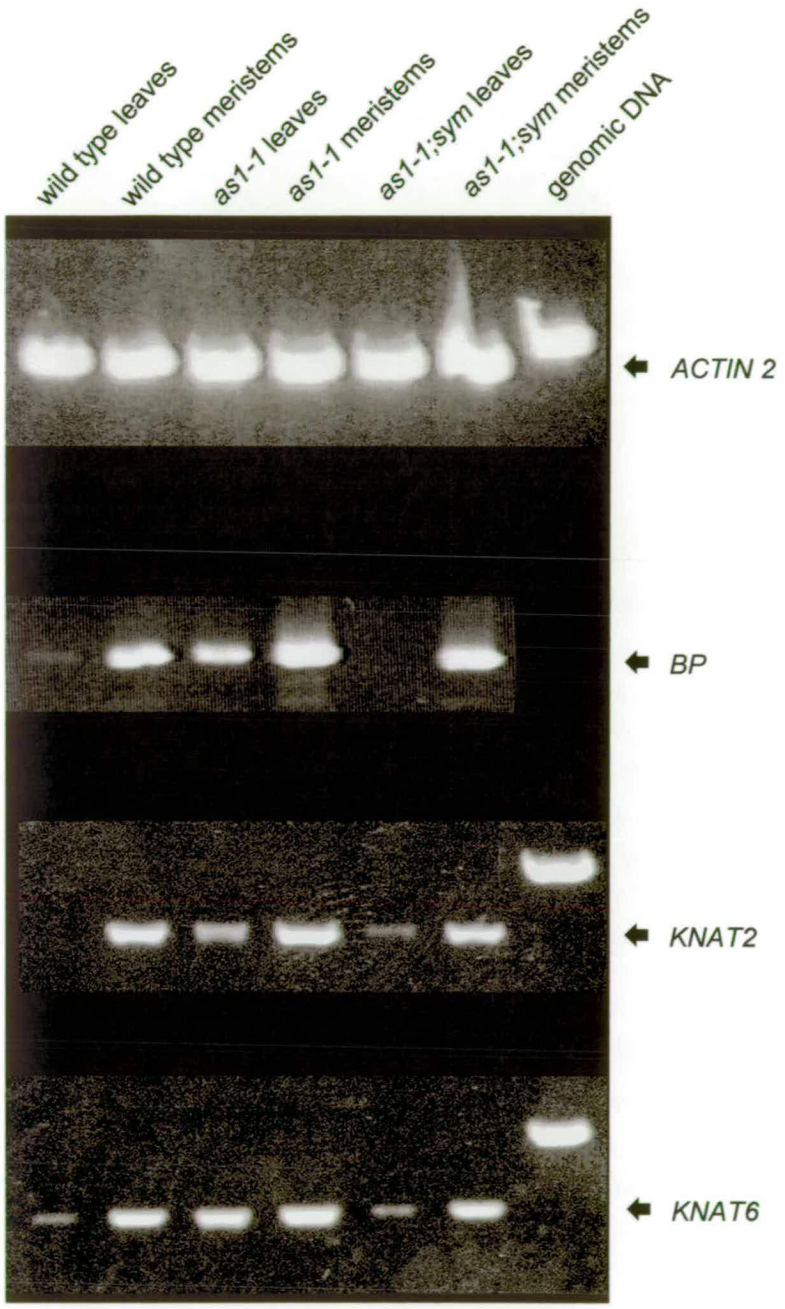


Figure 4.7 Levels of *knox* misexpression in *as1-1*, and *as1-1,sym* leaves by RT-PCR.
Wild type plants show little or no expression of *knox* genes in lateral organs, but strong expression in the meristem. *as1-1* mutants show normal *knox* expression in meristems, but misexpression in lateral organs. *as1-1;sym* plants despite the absence of a functional *AS1* have *knox* expression patterns similar to wild type plants.

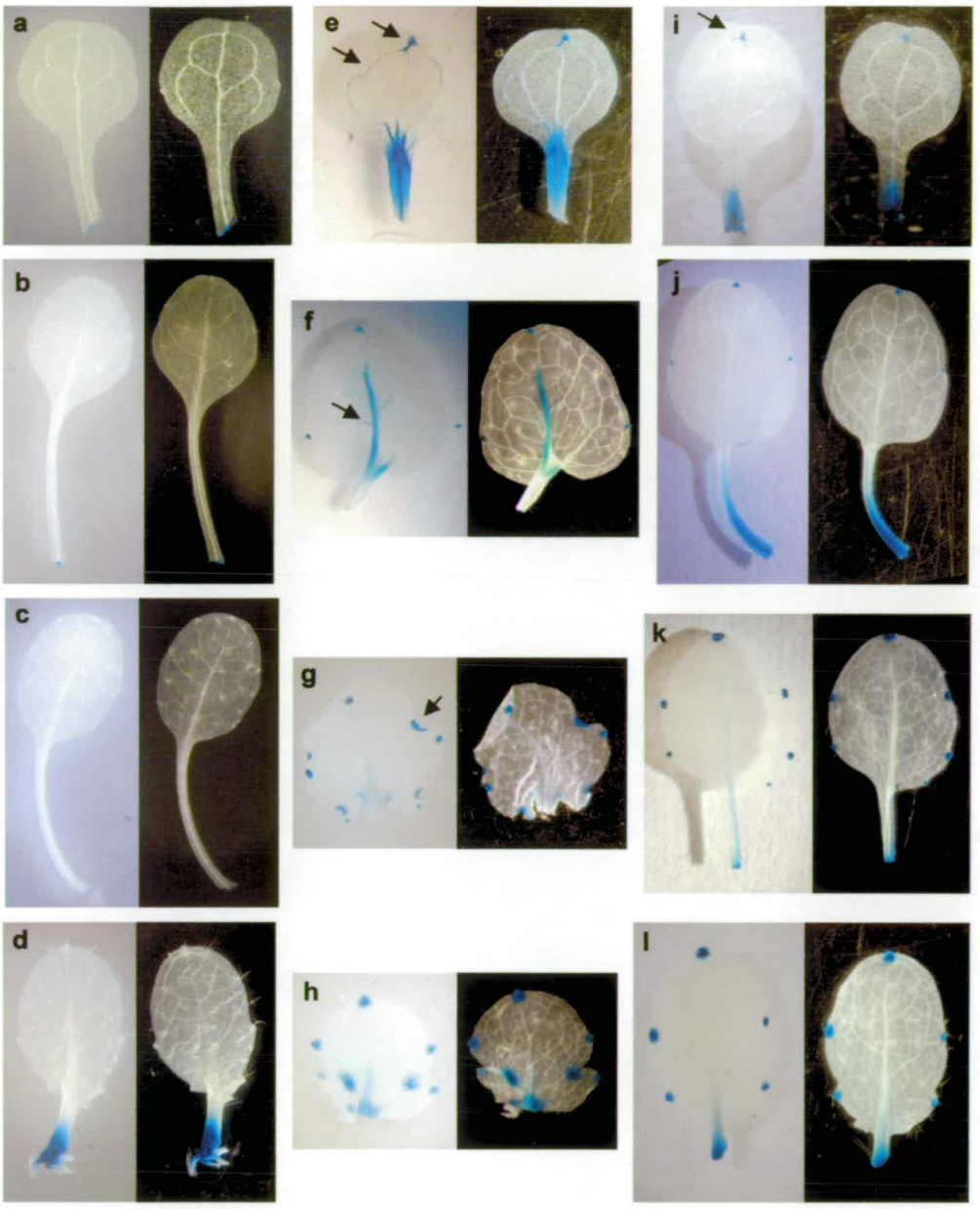


Figure 4.8 Localisation of p*BP::GUS* expression in cotyledons and first, third and fifth leaves in *as1-1,sym* and *as1-1* mutant backgrounds.

a-d Wild type. **e-h** *as1-1* **i-l** *as1-1 sym*. In cotyledons (**a**, **e** and **i**) GUS staining is observed in wild type where hypocotyl tissue is present at the base of the cotyledon, in *as1-1* throughout the petiole hydathode and vasculature (arrows). In *as1-1 sym* the petiole staining is restricted to the proximal half. Hydathodes are stained, but the vasculature is not. First and third leaves of wild type lack *GUS* expression. In *as1-1* the petiole, midrib, hydathodes and sinuses (arrow) are stained. In *as1-1 sym,* hydathodes and the proximal half of the petiole are stained. In *as1-1* and *as1-1 sym*, staining is as first and third leaves. In wild-type however, *GUS* is expressed a small way into the petiole.

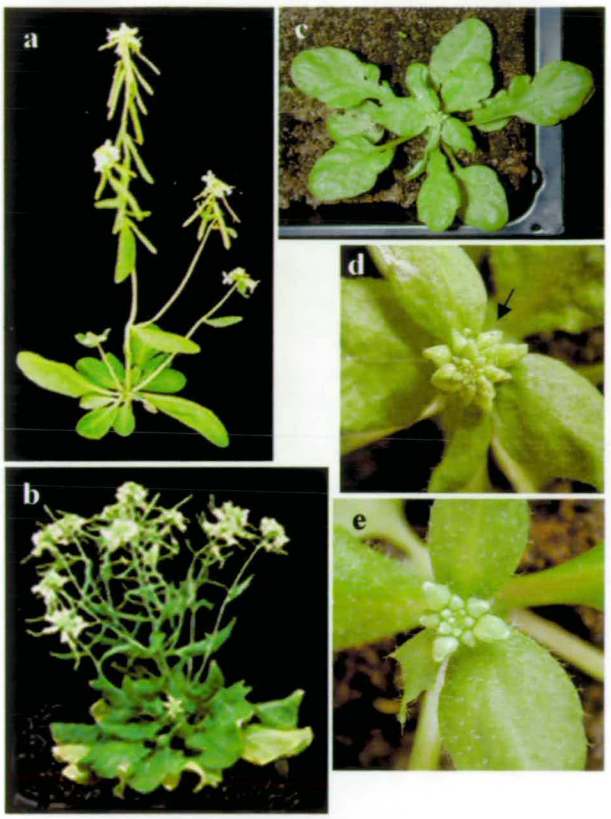


Figure 4.9 *bp* in an *as1-1 sym* mutant background.
a *bp-1* mutant. Flowers and siliques point down. **b** *as1-1 bp-1* mutant. Additive phenotype is demonstrated by development of an *as1* mutant rosette, and *bp* mutant inflorescence. **c** *as1-1 sym bp-1* triple mutant vegetative phenotype. *as1 sym*-like rosette. **d** Inflorescence of *as1-1 sym bp-1* triple mutant. Downward pointing flower buds (arrow) indicate a *bp*-like inflorescence architecture. **e** Wild type flower buds point upwards. (**a** taken from Ha, *et al.*, 2003)

4.1.9 *SYM* might not act redundantly with *BP*

bp as1 mutants show an additive phenotype (Byrne *et al.*, 2002), suggesting that ectopic *BP* expression is not responsible for the phenotype of *as1* mutant organs. Because *SYM* is required for ectopic *BP* expression in *as1*, it was expected that leaves of *bp*, *as1-1* and *sym* triple mutants would resemble *as1 sym* mutants. Because *SYM* is also needed for expression of *KNAT2* and *KNAT6*, a more severe *knox* loss-of-function phenotype (e.g. loss of SAM activity) was also considered a possibility.

An F2 population was generated from the cross of *bp* to *as1-1 sym*. Amongst the plants with *bp* mutant inflorescences that did not have the characteristic *as1* rosette, almost one quarter were expected to be suppressed *as1* mutants due to linkage to *sym*. Seven plants with this phenotype were genotyped by sequencing the *AS1* locus. Two were found to be *as1-1* homozygotes, two were wild-type *AS1* homozygotes and three sequences became unreadable at the site of the *as1-1* mutation (a single nucleotide deletion) suggesting that they were *as1*/+ heterozygotes. Some plants were also observed with small asymmetric lobes, as observed in some *as1 sym* double mutants (Fig. 4.9). However, the *as1 sym bp* mutants had the *bp* mutant phenotype, suggesting that *SYM* does not act redundantly with *BP*.

4.1.10 *sym* and *as2*

AS2 works at a similar position in development to *AS1*, probably because its protein product acts as a heterodimer with AS1 (Iwakawa *et al.*, 2002a; Semiarti *et al.*, 2001). *as2* mutants also have a very similar phenotype to *as1*, and like *AS1*, *AS2* expression is excluded from the developing meristem by *STM* and *as2* is a suppresser of the strong *stm-1* mutant phenotype (Byrne *et al.*, 2002).

To determine a possible genetic interaction between *as2* and *sym*, *as1-1 sym* double mutants were crossed to plants carrying the *as2-1* mutation (Antwerpen ecotype). F1 plants appeared as wild-type and an F2 population of 631 plants was analysed for segregation. Three sixteenths (19%) of the progeny were expected to be *as2* homozygotes. If *sym* suppressed both *as1* and *as2* semi-dominantly, almost all the *as1* mutants and three quarters of the *as2* mutants were expected to show suppression of the *as* phenotype and 3/64 of the total F2 (~5%) to have a strong *as* phenotype. Alternatively, if *sym* was unable to suppress the *as2* phenotype, then more plants – either ¼ (25%) or 3/16 (18%) depending on whether suppression of *as1* was epistatic to suppression of as2 – were expected to have a strong *as* phenotype. Plants with the *as1-1* phenotype comprised 1.74% of the F2 along with 138 other plants with strong *as* phenotypes (21.87 %). These plants were potential *as2-1* mutants and *as1-1 as2-1* double mutants as the double mutant phenotype has previously been described as *as2*-like (Serrano-Cartagena *et al.*, 1999). The remaining plants, totalling 482 (76.38 %) were grouped together as either

wild-type, or suppressed *as* mutants. The frequency of strong *as* phenotypes suggested that *sym* was unable to suppress the *as2* phenotype. However, phenotypic evidence suggested the *sym* mutation is capable of suppressing *as2*. Plants were observed with large laminae but which had occasional deep serrations characteristic of *as2* (Fig. 4.10). Insufficient time was available to confirm the genotypes of these plants. A possible reason for inconsistencies in phenotypic data is differences in ecotype. *as2* mutants, have been shown to have different phenotypes in different genetic backgrounds (Semiarti *et al.*, 2001).

4.1.11 *se* and *sym* modify the *as1-1* phenotype in similar ways

The *SERRATE* (*SE*) gene encodes a C_2H_2-type zinc-finger, implicated in maintaining target genes in a repressed state by chromatin remodelling (Prigge and Wagner, 2001). *se* single mutant have reduced and serrated leaves (Fig. 4.11; Clark *et al.*, 1999). Evidence suggests that like *sym*, *se* is a suppresser of *as1* as in *as1-1 se* double mutants, proximal-distal expansion of leaf lamina is restored (Fig. 4.11) and misexpression of *knox* genes in leaves is reduced. In order to determine the effect of the *sym* mutation in a *se* mutant background, *se* was crossed to *as1-1 sym* and an F2 population of 325 plants analysed. Due to linkage of *as1* to *sym* the presence of *as1* mutants (8 plants) and *as1 se* double mutants (2 plants) was low (3% and 0.6%, respectively). Segregation of plants with known *se* mutant phenotypes was lower than the expected 25%,

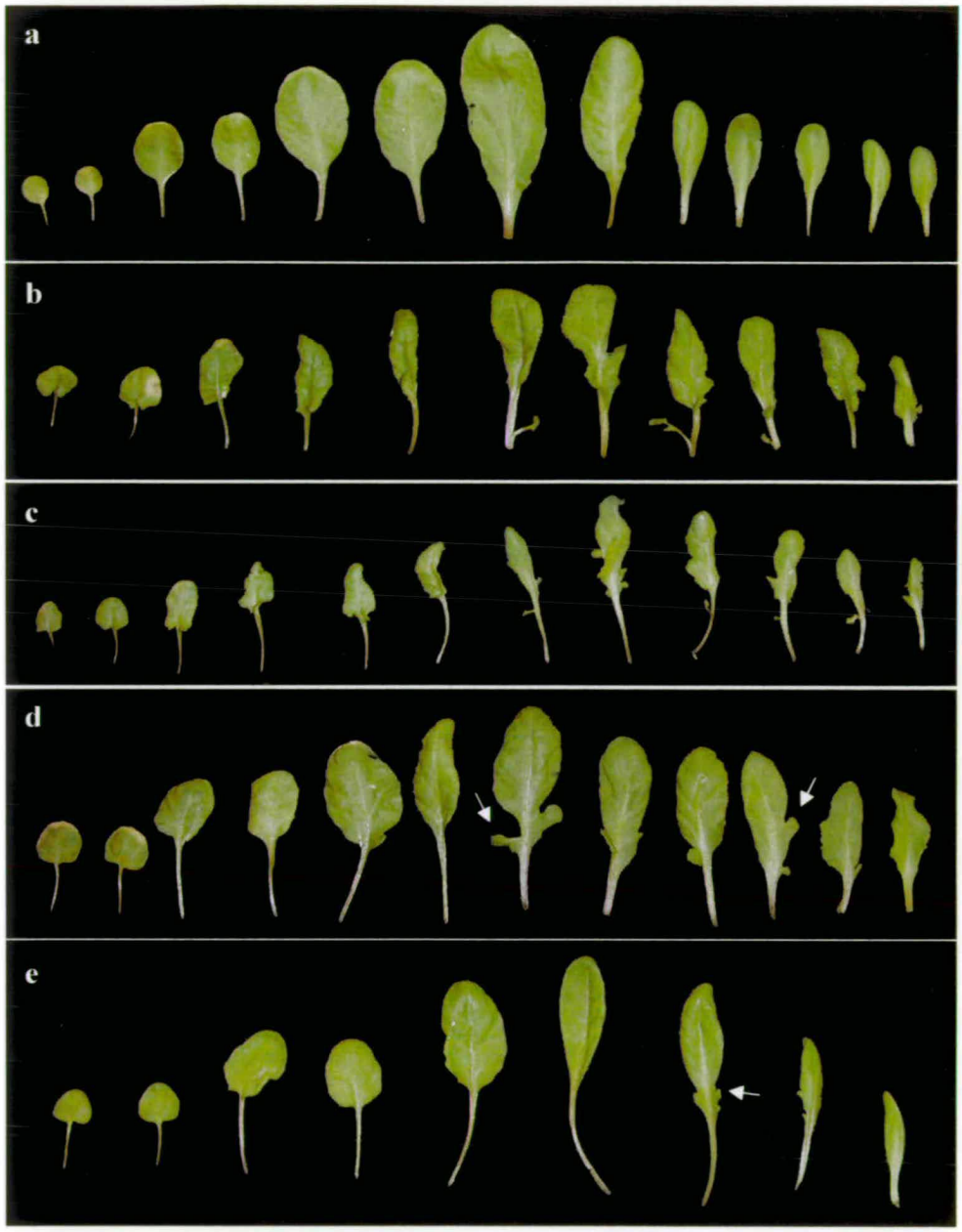


Figure 4.10 *as2-1* mutants compared to plants with suppressed *as2* phenotype.
a Wild type. **b and c** *as2-1* mutants from an F2 population segregating *as1-1*, *sym*, and *as2-1*. In **b** leaves are larger and broader than **c**, where narrow leaves with several serrations predominate. **d and e** Plants from the *as1-1 sym* x *as2-1* population with phenotypes suggestive of suppressed *as2*. In **d**, older leaves have deep serrations (arrows) close to the meristem and are predominantly asymmetric in shape. **e** Older leaves are narrow with small serrations (arrow).

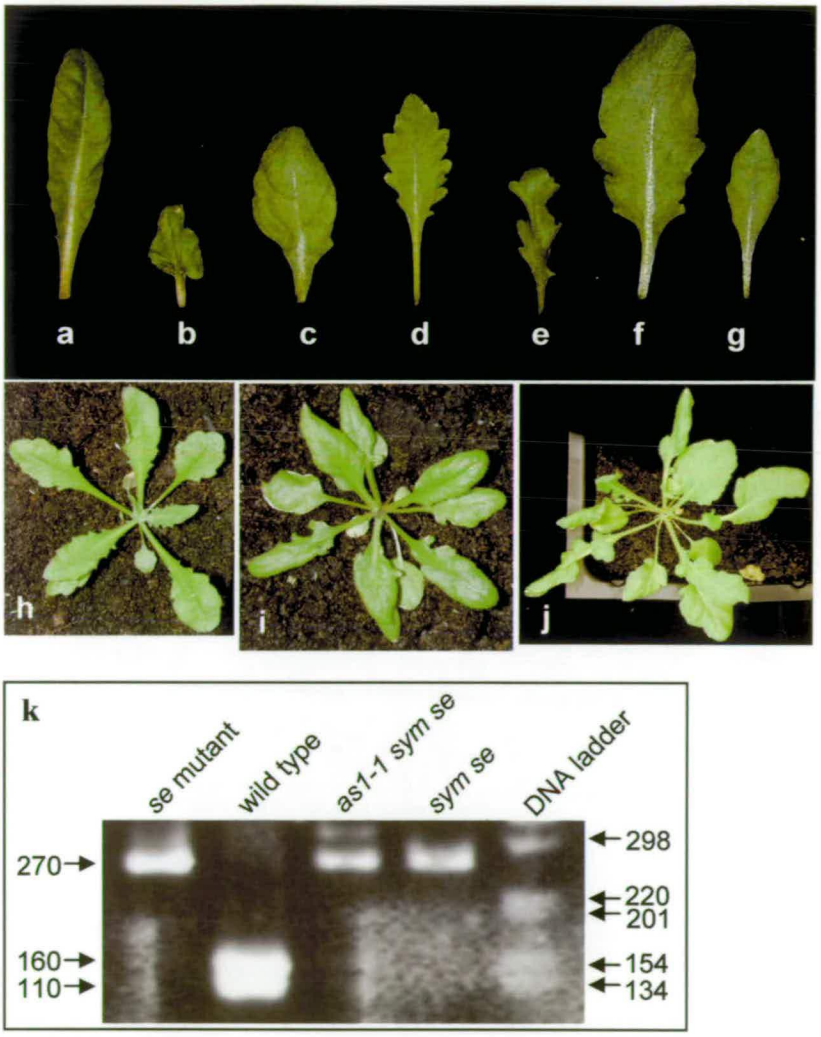


Figure 4.11 *sym* suppresses *se*.
a wild type. **b** *as1-1*. **c** *as1-1 sym*. **d and h** *se*. plants have reduced and serrated leaves. **e and i** *as1-1 se*. Deeper lobes but increase in expansion of lamina along the proximo-distal axis than *as1* single mutants. **f and j** *as1-1 sym se*. **k** Genotypes of plants in **f**, **g** and **j** at the *se* locus. A CAPS marker cleaves wild type but not *se* mutants.

occurring at 18% (56 plants; ~$^3/_{16}$) suggesting that *sym* was modifying the *se* phenotype in a different manner to *as1*. A novel phenotype was observed in 6% of progeny (22 plants; ~$^1/_{16}$), suggesting that these plants were *as1 sym se* triple mutants. These plants had serrations arranged in a less symmetric manner than described for *se* mutants and the leaf morphology was quite varied, possibly because of the semi-dominance of *sym* or other differences in genetic backgrounds. Some leaves formed fairly normally, with the exception of the serrations, which were generally more severe close to the stem. Other leaves on the other hand had deep lobes, more characteristic of the *as1* mutant phenotype but with greater expansion of the proximal-distal axis.

The cross yielded one other novel phenotype at 0.6% (2 plants) - a proportion that equalled that of *as1-1 se SYM*$^+$ plants, suggesting that it represented *sym se* double mutants. These plants had smaller leaves than wild-type and completely lacked serrations, suggesting that *sym* suppresses the serrated nature of *se* mutant leaves. It was not possible to determine whether *sym* acts upstream or downstream of *se* as the other notable aspect of the double mutant phenotype, leaf size, did not indicate an epistatic interaction. One explanation for the interaction of *se* and *sym* is that both are likely to be targets of *AS1* suppression since in *as1 sym* double mutants and in *as1 se* double mutants (Ori, *et al.*, 2000) *knox* expression in leaves is lower than *as1* single mutants, that is, both are required for strong *KNOX* misexpression in *as1* mutants, suggesting that they act at a similar position in the exclusion of *KNOX*

expression from lateral organs, although this contradicts Ori *et al.*, 2000, who suggest a role for *SE* downstream of *knox* genes.

In order to confirm the *SE* genotypes of the various phenotypic classes, a CAPS marker was designed. PCR primers were used to amplify a 270 bp section of the *SE* gene. The PCR fragment was then subjected to cleavage by the restriction enzyme *Bst YI* which in wild-type cuts to give 110 bp and 160 bp fragments at a site that is absent from the mutant *se* allele. The genotypes at the *se* locus for *sym se* and *as1-1 sym se* were confirmed (Fig. 4.11).

4.1.12 *SYM* has a role in the meristem

as1-1 stm-1 double mutants initiate and maintain a functional meristem despite the fact that *stm-1* single mutants do not. *STM* is required for the exclusion of *AS1* from the SAM and in the absence of *AS1* the role of *STM* in meristem initiation and maintenance becomes redundant (Byrne *et al.*, 2000). This is likely to reflect the expression of other *knox* genes in the absence of *AS1* activity. In order to determine a role for *SYM* in this genetic pathway, *as1-1 stm-1* double mutants were crossed to *as1-1 sym* double mutants. In the F2 population, 21% of plants were observed to have a strong *stm* phenotype (no functional meristem) despite the presence of the homozygous *as1-1* mutation in both parental lines, and therefore an absence of AS1 activity in all of the F2 population. *as1* and *as1 sym* mutants occurred at 25%, and 54%, respectively (Fig. 4.12). This suggests a role for *SYM* in activation of factors

Figure 4.12 *sym* is required for a functional SAM in *as1-1 stm-1* double mutants.
a *stm-1* mutant. Plants do not form an SAM. **b** *as1-1 stm* double mutant. A shoot apical meristem is formed and maintained. **c** *as1-1 stm-1 sym* triple mutant. No SAM is initiated suggesting a redundant role in the meristem for *SYM.* **d** *as1-1 sym stm-1* triple mutant showing weaker *stm*-like phenotype. A meristem is initiated (arrow), but not maintained. (**b** taken from Byrne, *et al.*, 2000.)

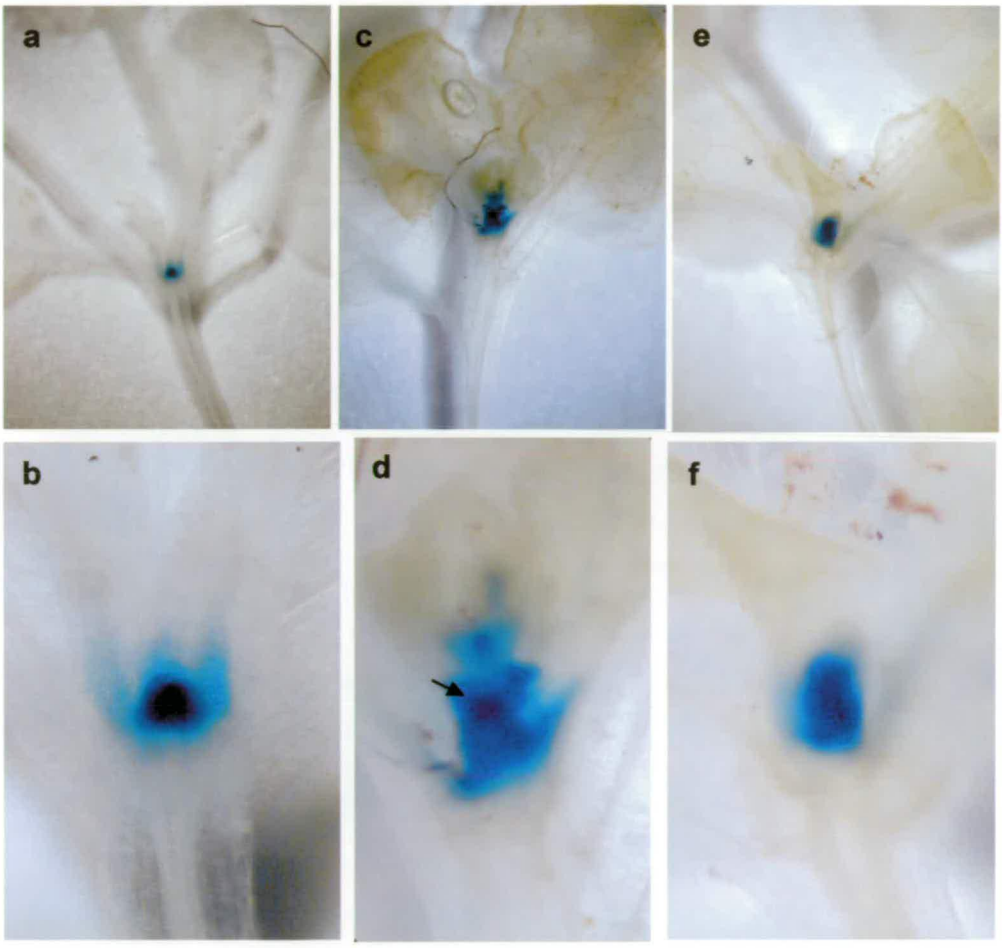
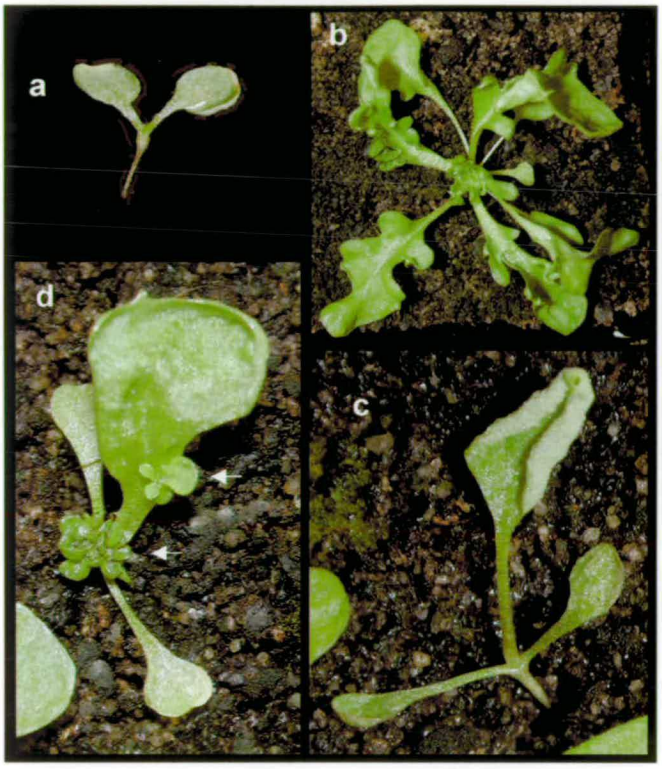


Figure 4.13 Localisation of p*STM::GUS* expression in the apex of *as1-1 sym* double mutants.

a and b wild-type. **c and d** *as1-1*. **e and f** *as1-1 sym*. **a**, **c** and **e** show *GUS* expression from the *STM* promoter occurs only at the apex in all three genetic backgrounds. In **b** Strong GUS staining is present only at the shoot apical meristem. In **d** staining occurs in the shoot apical meristem (arrow), and in lateral organs in tissue close to the apex. In **f** the area of GUS staining is larger than wild type, but stain is absent from lateral organs. Two explanations are reasonable. Either the meristem is larger, or expression from the *STM* promoter extends into the rib zone in *as1 sym* double mutants.

Figure 4.14 *stm-1 35S::BP* double mutants produce a functional meristem.
a *stm-1* mutant. **b**. *35S::BP* mutant. Plants show extreme lobeing of the lamina. **c** *35S::BP stm-1* plant early in development. Plants show no fusion of the cotyledons characteristic of *stm-1* plants and develop an organ where the meristem normally forms. **d** A new meristem is initiated and maintained from the base of the ectopic organ and occasionally from the leaf (arrows).



that act redundantly with *STM* in shoot apical meristem initiation and maintenance.

4.1.13 Reporter gene analysis suggests changes in *STM*-expressing regions at the shoot apex

As an interaction had been uncovered between *STM* and *SYM*, changes in *STM* expression were investigated in *as1-1 sym* double mutants. *as1-1* mutants carrying p*STM::GUS*, a vector which allows visualisation of the *STM* expression domain by expression of the *UidA* (GUS) gene from the *STM* promoter, were crossed to *as1-1 sym* double mutants. F2 plants carrying the transgene in wild-type, *as1* and *as1 sym* backgrounds were compared (Fig. 4.13). Wild-type plants accumulated GUS stain only at the shoot apex as expected. *as1-1* plants also demonstrated staining at the SAM but also ectopically in regions of lateral organs close to the SAM. Ectopic *STM* expression at low levels in *as1* mutants has been described for young leaves (Semiarti, *et. al.*, 2001), so staining in these regions is consistent with this observation. In *as1-1 sym* double mutants staining was not observed in lateral organs in a manner consistent with a requirement of *SYM* for ectopic *knox* expression in lateral organs of *as1* mutants. However, the staining pattern was markedly different from wild-type. Expression covered a larger domain which can be explained in one of two ways. The first possibility is that the area of *STM* expression extends into the rib zone in *as1 sym* double mutants. This would be consistent with partial suppression of ectopic *STM* expression

by *sym* in an *as1* mutant background. The second possibility is that *STM* expression does not extend outside the meristem but that the size of the meristem is altered in *as1 sym* mutants. This has been described for *se* mutants, and as *SYM* and *SE* are proposed to act similarly in plant development (Ori, *et. al.*, 2000), it is an intriguing possibility.

4.1.14 *BP* can act redundantly with *STM* in meristem initiation and maintenance when released from control by *AS1*

Other factors act redundantly with *STM* in meristem initiation and maintenance indicated by phenotypes of *as1-1 stm-1* mutants (Byrne *et al.*, 2000) as discussed above, and by *as1-1 stm-1 bp-1* triple mutants which appear like strong *stm* single mutants - i.e. they lack a functional SAM. This suggested that *STM* acts redundantly with *BP* and that ectopic expression of *AS1* in the *stm* mutant SAM represses *BP* preventing it maintaining the SAM (Byrne *et al.*, 2002). *35S::BP stm-1* plants were therefore expected to initiate and maintain a functional meristem as *BP* would no longer be subject to control by *AS1*. F2 seedlings from the *35S::BP* x *stm-1/+* cross were screened on kanamycin plates for the *35S::BP* transgene and then transferred to soil. In families that had inherited the *stm-1* allele, a quarter of the survivors developed an organ where the meristem normally forms. However these plants went on to produce a functional meristem from the junction of the cotyledons and the ectopic organ (Fig. 4.14) suggesting that *BP* can act redundantly with *STM* in meristem initiation and maintenance. Aside from

the role it has in organising inflorescence architecture, *BP* also appears to have different roles than *STM* in SAM organisation. In the absence of *STM*, *BP* expression through the CZ (from which it is normally absent) initially causes initiation of organ fate, a feature that also appears in weak *stm* mutations. This may be as a result of PZ characteristics occurring in the CZ.

4.1.15 Differences between *as1* and *35S::BP* mutations

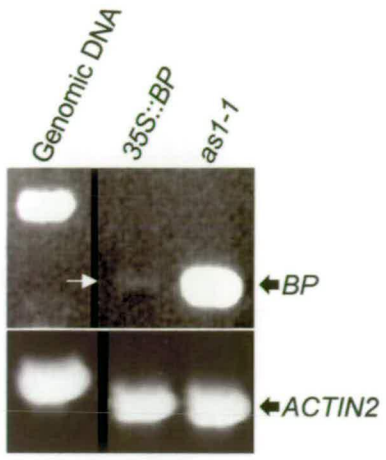
35S::BP mutants are phenotypically different from *as1* mutants, despite common features such as lobeing. In order to determine whether the different phenotypes could be associated with different expression levels of *BP*, leaves of *as1-1* mutants and *35S::BP* plants were subjected to RT-PCR to compare levels of *BP* mRNA. Interestingly, *as1* plants had a far higher level of *BP* mRNA than *35S::BP* plants, despite the strength of the constitutive *35S* CaMV promoter (Fig. 4.15). Because the factors downstream of *AS1* that normally regulate *BP* are unlikely also to act on the *35S* promoter, this suggests that regulation of *BP* by *AS1* might be mediated by post-transcriptional mechanisms.

4.1.16 *as1*, *sym* and *pinhead*

The *PINHEAD* locus encodes an ARGONATE family protein implicated in PTGS, a mechanism for post translational down-regulation of target genes (Fagard *et al.*, 2000). Additionally, *pnh* mutants look phenotypically similar

Figure 4.15 Accumulation of *BP* transcript in leaves of *35S::BP* and *as1-1* mutants.

BP transcript in *as1-1* mutant leaves is strong. In *35S::BP* mutants however, accumulation of transcript is on the edge of detection (arrow). *ACTIN2* transcript in both samples indicates that the samples are comparable.



to weak *stm* mutants and *35S::BP stm-1* double mutants, i.e. a lateral organ is developed where the SAM normally forms. During early stages of embryogenesis *STM* expression is unaffected in *pnh* mutants, suggesting that the shoot meristem programme is initiated correctly. In later embryo stages expression of *STM* is downregulated in the centre of the apex and becomes confined to a small group of lateral cells. This downregulation of CZ *STM* expression is a likely explanation for why *zll* seedlings mimic weak *stm* mutants and is probably a result of loss of the SAM (Moussian *et al.*, 1998). Despite down regulation of *STM* in the CZ of the meristem, *zll-3* mutants are not rescued by *as1-1* (Barley, 2001) suggesting that *PNH* is required for expression of other genes required for maintenance of the SAM. A small population of plants were sown from an *as1-1 zll-3/+* parent. The progeny segregated 5 *as1-1* mutants and 3 *as1-1 zll-3* mutants, which developed an organ where the SAM normally forms (Fig. 4.16). This additive phenotype suggested a lack of interaction between *AS1* and *PNH*, at least in early stages of development. Because the effects of *sym* in the *stm as1* SAM suggested a role for *SYM* in promoting SAM activity it might potentially interact with processes involving *PNH*.

To determine whether *SYM* interacts with *PNH*, *zll-3/+* plants were crossed to *as1-1 sym* mutants. An F2 population of 89 plants from a single parent was analysed. Sixty plants (67.42%) were of wild-type phenotype and these presumably included *as1-1 sym* double mutants. Eighteen *as1* mutants (20.22%) segregated in the F2, corresponding to just over $^{3}/_{16}$ths suggesting

that, like the background in which it was isolated, *sym* acts recessively in this background. Six plants (6.74%) developed an organ where the SAM normally forms. All *pnh* mutant alleles demonstrate variable penetrance, particularly at the transition to vegetative development, a value for which has not been described for the allele used. However, since a large percentage of the plants that presumably carry the homozygous *zll-3* mutation were able to continue to vegetative development, suppression of this aspect of the *pnh* phenotype by a mutation present in the *as1 sym* parental line cannot be ruled out. Since *as1* does not act as a suppresser of *pnh*, the reduced number of plants visibly carrying the *zll-3* allele may be caused by *sym*. Five plants with a novel phenotype were also found in the F2 population (5.61 %; Fig. 4.16). These plants were likely to carry both *zll-3* and *sym* mutant alleles. The *AS1* sequence was determined for all five and all were found to be homozygous for the *as1-1* mutation. Since *AS1* and *SYM* are linked, these plants were also likely to be *sym* homozygotes. However because of linkage, it was not possible to determine whether the *as1-1* mutation was also required for the novel phenotype. The novel phenotype included development of a rosette of leaves which were narrow, extremely wrinkled and showed severe abaxial curling. Development of lateral organs did not follow the normal phylotactic pattern, and elongation between nodes was variable. Genotyping at the *PNH* locus was not attempted as the mutation responsible for the *zll-3* allele used in the cross has not been determined.

Figure 4.16 Rosette phenotype of *as1-1 sym zll-3* triple mutant.

a *zll-3* mutant develops an organ at organ at the juncture of the cotyledons. **b** *as1-1 zll-3* double mutant has shorter petioles on cotyledons and rough lamina surface like *as1* single mutants. An organ is developed where the SAM normally forms as in *pnh* mutants. The phenotype therefore suggests addition. **c** In *as1-1 sym zll-3* triple mutants, a full rosette is developed, but the leaves are small, narrow with severe adaxial curling.

4.2 DISCUSSION

4.2.1 Ectopic *knox* expression alone cannot explain the *as1* mutant phenotype

One of the key roles of *AS1* in *Arabidopsis* development is exclusion of the expression of genes associated with the SAM in leaves and other lateral organs. In the absence of AS1 activity, genes of the *knotted1* family of homeobox genes become ectopically expressed (Ori, *et. al.*, 2000; Semiarti, *et. al.* 2001). Perhaps the most striking phenotype of *as1* mutants is observed in the strongest known allele, *as1-magnifica*, which develops ectopic meristems on leaf margins at the base of lobes providing a graphic illustration of expression of genes required for meristem fate occurring in lateral organs. This same feature of ectopic meristems is observed in plants with a functional *AS1* gene, when *BP* is constitutively expressed from the *35S* promoter, as are other features of the *as1* phenotype such as heart shaped leaves and lobes (Chuck *et al.*, 1996).

knox genes are however the only known genes involved in specifying meristem fate that are mis-expressed in *as1* mutant lateral organs and, if *knox* activity is reduced, *as1* mutant leaves retain their defects - *as1-1 bp* double mutants have the *as1* rosette phenotype (Byrne, *et al.*, 2002) as do *as1-1 knat2* and *as1-1 stm-1* double mutants (Byrne, *et al.*, 2000). No *knat6* mutant is currently available with which to test the role of *KNAT6* mis-expression in *as1* leaves. The current evidence therefore suggests that there are other factors involved in causing the *as1* mutant phenotype.

The *sym* mutation was identified in a modifier screen as a complete suppresser of *as1-1*, and since this has not been achievable by reduction of *knox* expression, *sym* identified a potential target of *AS1* involved in leaf morphogenesis and *knox* repression.

4.2.2 Changes in *knox* expression in *as1-1 sym* double mutants

Exclusion of *knox* proteins from leaf founder cells is believed to be important in the acquisition of leaf fate (Smith *et al.*, 1992; Jackson *et al.*, 1994). Ectopic expression of *knox* genes in leaves of different plants results in dramatic tissue transformations, including ectopic meristematic activity (Sinha *et al.*, 1993; Lincoln *et al.*, 1994; Schneeberger *et al.*, 1995; Chuck *et al.*, 1996; Byrne, *et al.*, 2000). *as1* mutants strongly mis-express *knox* genes in lateral organs, but in the absence of SYM activity, RT-PCR and reporter gene expression analyses indicates that ectopic *knox* expression is reduced. Occasional lobes close to the stem in *as1-1 sym* mutant leaves may reflect low levels of ectopic *knox* transcript as observed for ectopic *BP* expression at the base of petioles in *as1 sym* mutants.

SYM therefore has a role in up-regulation of *knox* genes, as in its absence the levels of *BP*, *knat2* and *knat6* transcripts are reduced in *as1* mutant leaves. This is likely to reflect transcriptional control because mis-expression of the GUS reporter gene from the *BP* promoter is also reduced in *as1 sym* relative to *as1*. SYM is therefore likely to be an upstream activator of *BP*, *knat2* and *knat6*. *AS1* would then exclude *knox* expression from lateral organs by

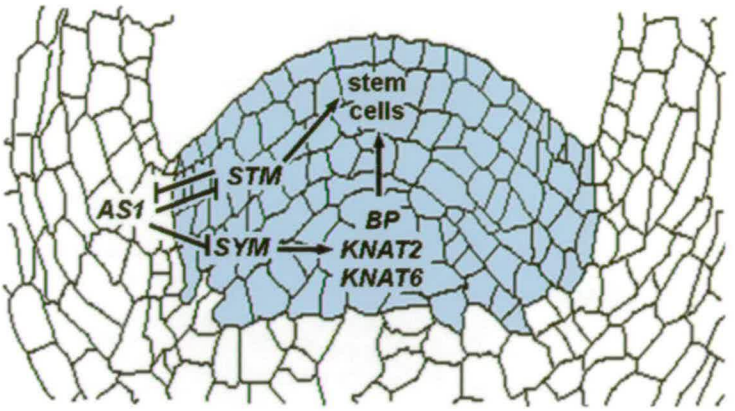


Figure 4.17 Model of genetic interactions at the shoot apex.
STM is required for maintenance of the stem cell population at the centre of the apex. Part of this function is the exclusion of *AS1* from the SAM. *AS1* is involved in specifying lateral organ fate and one of the ways in which this occurs is by exclusion of *STM* and the *knox* genes *BP*, *KNAT2* and *KNAT6* that act redundantly with *STM* from the lateral organs. Activation of *BP*, *KNAT2* and *KNAT6* requires *SYM* in a redundant manner. (figure adapted from Haeker and Laux, 2001.)

repressing *SYM* expression in organs (Fig. 4.17). Loss of *SYM* activity has no phenotypic effects in an *AS1*$^{+}$ background, *SYM* is likely to act redundantly with other genes which might also be targets of down-regulation by *AS1*. A reason for the persistence of low levels of *knox* expression in *as1-1 sym* double mutant leaves may be activation of *BP*, *KNAT2* and *KNAT6* by factors that act redundantly with *SYM*. Redundant gene action in meristem signalling is demonstrated by the *CUC1* and *CUC2* genes which are thought to be involved in setting meristem boundaries (Takada *et al.*, 2001). Both are required for expression of the *knox* gene *STM*. In the absence of only one of the *CUC* genes, *STM* transcript accumulates normally (Aida *et al.*, 1999).

4.2.3 An interaction between *sym* and *as2* is likely

AS1 has been shown to physically interact with *AS2* (Iwakawa *et al.*, 2002a). Both have similar mutant phenotypes, both are expressed in lateral organs, but not in the SAM, and both mutants have been shown to mis-express *knox* genes in lateral organs and to suppress *stm* mutants (Byrne *et al.*, 2002; Ueno *et al.*, 2002). The only notable difference between the two genes is expression patterns within the lateral organs. *AS1* is expressed throughout lateral organs (Byrne *et al.*, 2000), whereas *AS2* is expressed adaxially, suggesting that although the two genes are likely to act together in *knox* repression, that they might also likely to act independently in other developmental roles. These differing roles may be highlighted in a *sym* mutant background since *sym* is a dominant suppresser of *as1*, but phenotypes of likely *as1 as2 sym* triple

mutants suggest that *sym* is a recessive suppresser of *as2*. The triple mutant results are, however, difficult to interpret as segregation patterns from crosses did not fall into normal genetic ratios and were compounded by differing genetic backgrounds of the parental lines. However, characteristics of *as2* mutants were clearly visible in suppressed plants and these characteristics were not observed in populations of suppressed *as1*. An alternative interpretation would be that there is no interaction between *as2* and *sym* and this would highlight differing roles of the two *as* mutants. Genotype analysis of potential double mutants will be necessary to distinguish between these possibilities.

4.2.4 The function of *SERRATE* remains obscure

The *serrate* mutation has been described as an enhancer of the *as1* mutant phenotype. The basis of this interpretation is that the leaf lobes of the double mutant are larger and more numerous than *as1* single mutants and the sinuses have an increased capacity for generation of ectopic meristems (Ori *et al.*, 2000). *se* mutants also have a larger meristem which could be interpreted as a promotion of meristematic activity, although generation of leaves occurs more slowly (Clark *et al.*, 1999; Prigge and Wagner, 2001). A simpler explanation for the *as1-1 se* double mutant phenotype is that it represents suppression of the *as1* phenotype. *as1-1 se* double mutants have significantly reduced knox mis-expression in lateral organs, far greater proximal-distal leaf expansion and longer petioles than *as1*. A further doubt about the "enhancer" interpretation

is provided by co-suppressed plants that show phenotypes associated with a compromised meristem, indeed in extreme cases the meristem is not maintained. One explanation for the apparent contradictions in phenotypes is that the sinuses of *as1* se leaves take on characteristics of the meristem/lateral organ boundary. These are the only parts of wild-type leaves to express *knox* genes, show reduced growth and are able to produce (axillary) SAMs.

There are startling similarities between the ability of *se* and *sym* mutations to suppress the *as1* mutant phenotype. Both *as1 se* and *as1 sym* double mutants have restored proximal-distal growth of the lamina, develop petioles and show reduced *knox* mis-expression. This suggests that *SYM* and *SE* have similar roles. This view is also supported by the phenotype of *as1 sym se* triple mutants that develop large leaves with serrations or lobes close to the stem. These lobes are not as profound as those found in *as1 se* double mutants, nor are they as regular as *se* single mutants. The most likely explanation is further suppression of the *as1-1* phenotype by *se*, consistent with *SE* and *SYM* acting independently on common targets. *SYM* can therefore be placed at a similar position to *SE* in development, as an activator of *knox* expression.

SE encodes a zinc finger protein that, by homology, is thought to be involved in chromatin remodelling required for maintaining genes in a silenced state (Prigge and Wagner, 2001). *SE* might therefore be involved in maintaining *knox* gene repression in lateral organs. The apparent contradictions in the developmental roles of *SE* might also lie in SE's biochemical function – it is

likely to have multiple downstream targets involved in different developmental processes.

4.2.5 *SYM* has a redundant function in the shoot apical meristem

In the absence of activity of the *knox* gene, *STM*, genes that specify lateral organ fate become expressed throughout the apex of the *Arabidopsis* embryo, resulting in loss of stem cell initiation and maintenance (Byrne *et al.*, 2000; Byrne *et al.*, 2002). If the activity of lateral organ genes is reduced in the apex of *stm* mutants, a meristem is initiated and maintained as, for example, in *as1-1 stm-1* double mutants, and *as2 stm* double mutants. This suggests that *STM* acts to promote SAM activity in two ways – one of which involves repressing organ genes such as *AS1* and *AS2*, which when mis-expressed can repress other *knox* genes and prevent them contributing to SAM maintenance. One of the key factors in this pathway is *STM*'s fellow *knox* gene *BP*, with which it shares most homology. In triple mutant plants that lack *as1*, *stm* and *bp*, a functional meristem is not maintained, despite the reduction in lateral organ fate at the apex due to the absence of *AS1* (Byrne *et al.*, 2002). The role of *BP* as a redundant partner of *STM* was confirmed by complementation of the strong *stm-1* mutation by a p*35S::BP* transgene. Plants produced a functional meristem, but only after initially developing a leaf at the apex. The meristem was initiated from the site of the junction between the ectopic leaf and the hypocotyl. The ectopic leaf might have been the result of residual *AS1/AS2* expression at the apex or acquisition if peripheral zone

characteristics throughout the SAM (*BP* is normally expressed only in the PZ of the SAM where its promoter is active Lincoln *et al.*, 1994b).

Supporting evidence for a role for *SYM* in promoting *knox* gene expression comes from *as1-1 sym stm-1* triple mutant analysis. *as1-1 stm-1* double mutants normally produce a functional SAM because the absence of AS1 activity allows expression of *knox* genes. This is not the case with additional loss of SYM activity – *as1-1 sym stm-1* plants are unable to form an embryonic SAM and resemble strong *stm* mutants. A small number of plants can, however, initiate a meristem at a position where cotyledons have become fused – the area where the SAM would normally form. In these cases the SAM is not maintained. This contrasts with the *as1-1 stm-1 bp* triple mutant which is unable to form a SAM. One reasons for the difference might be that a higher level of *knox* expression in the presence of the *sym* mutation. This is consistent with the inability of *sym* to completely suppress the *knox* mis-expression phenotype of *as1* and the observation that more *knox* transcripts can be detected by RT-PCR in apices of *as1-1 sym* double mutants than in wild-type. Genetic analysis does however suggest that a decrease in overall *knox* expression is likely to be caused by the *sym* mutation and in the case of *STM*, reporter gene analysis (*UidA* driven from the *STM* promoter) indicated that the area of *STM* expression is likely to be altered in *as1-1 sym* double mutants relative to wild-type. In *as1-1 sym* double mutants *STM* reporter gene expression was absent from lateral organs as in wild-type, but not *as1* mutants where it extended into the base of petioles. However, a larger area of

expression was observed. This has two possible explanations (1) that *STM* expression had extended into the rib zone of the meristem from which it is absent in wild-type but present ectopically in *as1* mutants. This is consistent with the inability of the *sym* mutation to completely suppress *knox* mis-expression. (2) that the meristem itself has become larger, so *STM* is active within its normal, but larger, expression domain. Increases in overall meristem size have been observed in the *clavata* mutants which encode genes required for regulation of meristem size (Clark *et al.*, 1996; Clark *et al.*, 1993; Clark *et al.*, 1995; Kayes and Clark, 1998; Schoof *et al.*, 2000; Trotochaud *et al.*, 2000). However an increase in meristem size along the apical-basal axis is also observed in *serrate* mutants. Although the reasons for this remain obscure, it seems reasonable to suggest that *sym* might cause a similar defect as the two genes are proposed to have similar roles in regulating *knox* genes.

4.2.6 PTGS and plant development.

One problem with interpreting phenotypes resulting from mutations in the PTGS machinery is that many genes acting in different developmental processes might be mis-regulated in mutants. In the case of *PNH*, at least two roles are suggested by the *pnh* mutant phenotype - one in maintenance of the stem cells at the shoot apex (Lynn *et al.*, 1999; McConnell and Barton, 1995), and one in specifying dorsal cell fates in lateral organs (Rhoades *et al.*, 2002; Tang *et al.*, 2003).

Analysis of the *sym* mutant phenotype and interactions of the *sym* mutation suggest that *SYM* promotes SAM activity by promoting *knox* gene expression – a role similar to that proposed for *PNH*. However, *sym* and *pnh* (*zll-3*) mutations interact in an unexpected way. Whereas *zll*-3 plants produce no SAM, sym *zll-3* plants produce a SAM which develops a rosette. *sym* is therefore a suppresser of *pnh*, consistent with a role for *SYM* in negative regulation of meristem fate. However, suppression of the *pnh* phenotype might also reflect the pleitropic role of *PNH* in PTGS, as it seems able to promote both organ fate and meristem fate.

In terms of lateral organs, *pnh* mutants have leaves that curl adaxially (McConnell and Barton, 1995), whereas *as1-1 sym zll-3* triple mutants have leaves that curl abaxially. Curled leaves are generated as a result of differential lamina expansion between adaxial and abaxial surfaces. In *pnh* single mutants, curling is presumably an effect of loss of adaxial identity. As *as1-1 sym zll-3* triple mutants show abaxially curling, they suggest that *sym* is suppressing the *pnh* phenotype in leaves, as was observed in the SAM. As the biochemical function and expression pattern of *sym* are unknown is difficult to suggest in what way this apparent suppression of *pnh* is likely to act.

4.2.7 Candidate genes for *SYMMETRICA*

In the region of chromosome 2 to which *SYM* was mapped there are a number of good candidate genes. Given that *sym* and *pnh* mutations interact, and therefore that *SYM* might function in the RNA-induced silencing machinery,

one candidate is a putative RNA-binding protein. Various protein kinases and phosphatases are also candidates as these proteins have potential roles in signal transduction pathways. Meristem size, for example is maintained by a signal transduction pathway involving the CLV1 protein kinase and a protein phosphatase (Yu *et al.*, 2003; Yu *et al.*, 2000). The outstanding candidate, however, is a NAM-like gene closely related to *CUC2*. The *CUC* genes are required to set the boundaries for *knox* expression, and have been shown to positively regulate *knox* genes (Aida *et al.*, 1997; Aida *et al.*, 1999; Hibara *et al.*, 2002). One model is therefore that *SYM* encodes NAM-like function at the boundary between organs and the SAM from where it promotes *knox* expression in the remainder of the SAM, presumably via a signal and is repressed by *AS1/AS2* in leaves. In a preliminary investigation, full length mRNA from the candidate gene was not detected *as1-1 sym* mutants but was present in wild-type and *as1-1*. The NAM-like gene however was not found to be misexpressed in *as1* lateral organs as predicted. However the level of mis-expression might have been below the level of detection. Alternatively, the ectopic activity of *SYM* might not involve ectopic *SYM* transcription, as proposed for activation of *KNOX* genes by NAM-like functions.

Once the gene encoded by the *SYM* locus has been determined, it will be interesting to determine how the interactions occur between genes of such diverse biochemical function. *SYM* interacts with genes involved in chromatin remodelling (*SE*), and RNA induced silencing (*PNH*) as well as the homeobox

(*STM* and the other *knox* genes). This may lead to understanding of the changes in gene expression profiles responsible for the *as1* mutant phenotype.

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